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PRINCIPAL INVESTIGATOR: Wei-Zen Wei, Ph.D.

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Detroit, Michigan 48201

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Wei-Zen Wei, Ph.D.

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Detroit, Michigan 48201E-Mail: [weiw@karmanos.org](mailto:weiw@karmanos.org)8. PERFORMING ORGANIZATION  
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This study tests whether breast cancer can be eliminated by immunization with foreign peptides followed by delivery of peptides to tumors. We proposed to (1) establish an in vitro assay to measure tumor growth inhibition, (2) synthesize pro-peptides for activation at the tumor site by beta-glucuronidase, and (3) develop human CTL line and measure pro-peptide activity with human CTL. We tested two methodologies for synthesizing Flu MP58 pro-peptide. Condensation of Beta-glucuronide conjugated glycine to octapeptide ILGFVFTL was superior when compared to direct conjugation of beta-glucuronide to the nonapeptide GILGFVFTL. A human CTL line to MP58 was established by repeated stimulation of CD8 T cells with MP58 loaded antigen presenting cells. MP58 loaded T2 cells were lysed by CTL at E:T ratio of 10:1 - 2:1. The same CTL did not lyse T2 cells loaded with pro-peptide beta-Glu-MP58 unless beta-glucuronidase was added to the culture, further demonstrating liberation of active MP58 from the prodrug to mark tumor cells for CTL lysis. These results demonstrate that peptide prodrugs can be converted by beta-glucuronidase to active peptide and mark tumor cells for CTL mediated lysis.

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## INTRODUCTION

As described and approved in the last report, Task 2 of the project has been modified to facilitate the translation of the lab products to potential clinical trials. Therefore, Flu peptide MP-58 GILGFVFTL presented by human HLA-A2.1 rather than beta-galactosidase peptide TPHPARIGL presented by mouse K<sup>d</sup> is used for pro-peptide development. In this report, Task 3 is modified in order to measure MP-58 prodrug activity with human effector cells in vitro. The specific Tasks are :

- Task 1 Establish an in vitro assay to measure tumor growth inhibition by peptide specific T cells and pro-peptides
- Task 2 Synthesize and test the activity of pro-peptides to be activated by  $\beta$ -glucuronidase at the tumor site
  - (A) Synthesis of glucuronide derivatives of Flu peptide MP-58 GILGFVFTL presented by human HLA-A2.1.
  - (B) Measurement of peptide and pro-peptide binding to MHC.
- Task 3 Develop human anti-MP58 CTL line and measure pro-peptide activity with anti- MP58 CTL.

## BODY

**Task 1** Establish an in vitro assay to measure tumor growth inhibition by peptide specific T cells and pro-peptides  
Results from this task were detailed in previous progress reports. A paper describing this task has been published (Appendix A).

**Task 2** Synthesize and test the activity of pro-peptides to be activated by  $\beta$ -glucuronidase at the tumor site

- (A) Synthesis of glucuronide derivatives of Flu peptide MP-58 GILGFVFTL presented by human HLA-A2.1.
- (B) Measurement of peptide and pro-peptide binding to MHC.

Results from this task have been published (Appendix B).

Two methods for synthesis of prodrugs of MP58 **2a** (see appendix B) activated by beta-glucuronidase and comprising a self-immolative 3-nitrobenzyloxycarbonyl moiety were investigated. Reaction of beta-glucuronic acid glycoside of 4-hydroxy-3-nitrobenzyl alcohol (**3**) with N,N'-disuccinimidyl carbonate (DSC) followed by conjugation with AlaOme, Gly, Thr, Phe-Leu, or Leu-Arg gave carbamates **4a-4f**. Deacetylation of **4b** and **4e** with MeONa/MeOH gave beta-glucuronides **5b** and **5e**. Compound **5e** was converted to beta-glucuronic acid conjugate **6e** by the action of pig liver esterase (PLE). Compound **6e** is a substrate for beta-glucuronidase. Method of a direct introduction of the prodrug residue into MP58 GILGFVFTL (**2b**) failed. Alternately, glycine conjugate **5b** was activated to pentafluorophenyl ester **10**. Model coupling of **10** with Phe-Leu gave tripeptide conjugate ester **11a** which was hydrolyzed by PLE to uronic acid **12**. Condensation of **10** with octapeptide ILGFVFTL (**9**) gave prodrug precursor **11b**. Octapeptide **9** was prepared by de novo synthesis using a racemization-free

fragment coupling method. Ester hydrolysis with Ba(OH)<sub>2</sub>/MeOH gave the target prodrug **2a** which is a substrate for beta-glucuronidase. Prodrug **2a** does not bind to HLA-A2.1 of T2 human cells defective in major histocompatibility complex I (MHC I) – associated peptide processing. Addition of beta-glucuronidase restored the binding to the level observed with parent nonapeptide **2b** although higher concentrations of prodrug **2a** and enzyme were necessary.

**Task 3** Develop human anti-MP58 CTL line and measure pro-peptide activity with anti- MP58 CTL

A MP58 specific CTL line was established from peripheral blood monocytes of an A2.1 positive normal adult man by repeated stimulation with MP58 loaded antigen presenting cells. Dendritic cells were prepared from blood monocytes by culturing in AIM V medium supplemented with flt3 ligand, c-kit ligand, GM-CSF, TNF-alpha and IL-4 for 6 days in 10% autologous serum (Ferlazzo et al. 3597-604). Such DC were loaded with 40 ug/ml MP58 peptide in the presence of 3 ug/ml beta-microglobulin for 2 hrs at 37 degree. After three washes, DC were incubated with autologous CD8+ T cells in medium containing 10 ng/ml of IL-7. CD8 T cells were prepared by negative depletion of blood MNC which expressed CD20, CD4, CD14 and CD32, using magnetic beads. The antigenic stimulation was repeated with monocytes as the antigen presenting cells. Over 80% of MP58 loaded T2 cells were lysed by CTL at E:T ratio of 10:1 – 2:1. The same CTL did not lyse T2 cells loaded with pro-peptide beta-Glu-MP58. In the presence of beta-glucuronidase, 80% of T2 cells were lysed, demonstrating liberation of active MP58 from the prodrug to mark tumor cells for CTL lysis. These results demonstrate that immunogenic peptide prodrugs are converted to active peptide by beta-glucuronidase and mark tumor cells for CTL mediated lysis.

**KEY RESEARCH ACCOMPLISHMENTS**

1. An in vitro 3-D tumor growth inhibition assay was established to measure anti-tumor activity of peptide specific cytotoxic T cells (Appendix A).
2. Beta-Glu-MP58, pro-peptide of flu MP-58 GILGFVFTL, was synthesized. In the presence of  $\beta$ - glucuronidase, MP-58 peptide is released from the pro-peptide and bound to HLA-A2.1. (Appendix B).
3. A human CTL line to flu peptide MP58 was established. Anti-MP58 CTL lysed T2 cells loaded with pro-peptide beta-Glu-MP58 only in the presence of beta-glucuronidase, demonstrating liberation of active MP58 from the prodrug to mark tumor cells for CTL lysis.

**REPORTABLE OUTCOMES**

Wei-Zen Wei, Stuart Ratner, Terry Shibuya, George Yoo and Agnes Jani, Foreign antigenic peptides delivered to the tumor as targets of cytotoxic T cells. *J. Immunol. Method.* 258:141-150, 2001

Rewale, S., Hrihorczuk, LM., Wei, WZ and Zemlicka, J., Synthesis and biological activity of prodrug of class I major histocompatibility peptide GILGFVFTL activated by beta-glucuronidase. *Journal of Medicinal Chemistry.* 45: 937-943, 2002

## CONCLUSIONS

MP58 pro-peptide has been synthesized. Active MP58 peptide can be released by beta-glucuronidase to bind MHC I on target cells, marking them for specific CTL lysis. Prodrug of immunogenic peptide is a potential therapeutic agent for solid tumors which contain significant level of beta-glucuronidase.

## REFERENCE

Ferlazzo, G., Wesa, A., Wei, WZ. and Galy, A. "Dendritic cells generated from either CD34<sup>+</sup> progenitor cells or from monocytes differ in their ability to activate antigen-specific CD8<sup>+</sup> T cells." *Journal of Immunology* 163 (1999): 3597-604.

## APPENDICES

- A. Wei-Zen Wei, Stuart Ratner, Terry Shibuya, George Yoo and Agnes Jani, Foreign antigenic peptides delivered to the tumor as targets of cytotoxic T cells. *J. Immunol. Method.* 258:141-150, 2001
- B. Rewale, S., Hrihorczuk, LM., Wei, WZ and Zemlicka, J., Synthesis and biological activity of prodrug of class I major histocompatibility peptide GILGFVFTL activated by beta-glucuronidase. *Journal of Medicinal Chemistry.* 45: 937-943, 2002

### Reference List

Ferlazzo, G. et al. "Dendritic cells generated from either CD34<sup>+</sup> progenitor cells or from monocytes differ in their ability to activate antigen-specific CD8<sup>+</sup> T cells." Journal of Immunology 163 (1999): 3597-604.

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Appendix A

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## Foreign antigenic peptides delivered to the tumor as targets of cytotoxic T cells

Wei-Zen Wei \*, Stuart Ratner, Terry Shibuya, George Yoo, Agnes Jani

*Karmanos Cancer Institute, Wayne State University, 110 E. Warren Avenue, Detroit, MI 48201, USA*

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In addition the journal will publish articles on novel methods for analysing the organization, structure and expression of genes for immunologically important molecules such as immunoglobulins, T cell receptors and accessory molecules involved in antigen recognition, processing and presentation. Submitted full length manuscripts should describe new methods of broad applicability to immunology and not simply the application of an established method to a particular substance although papers describing such applications may be considered for publication as a short Technical Note.

The Recombinant Technology section will contain articles relating to modification by recombinant techniques of molecules of immunological interest; isolation of novel binding proteins by phage display; gene therapy; transfection; and expression. Immunology Protocols is a section providing detailed, step-by-step descriptions of new and established techniques in immunology. Articles on the molecular biological analysis of immunologically relevant receptor binding sites are also invited.

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## Foreign antigenic peptides delivered to the tumor as targets of cytotoxic T cells

Wei-Zen Wei\*, Stuart Ratner, Terry Shibuya, George Yoo, Agnes Jani

Karmanos Cancer Institute, Wayne State University, 110 E. Warren Avenue, Detroit, MI 48201, USA

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### Abstract

Cytotoxic T cells (CTL) are readily activated by immunogenic peptides and they exert potent anti-tumor activity if the same peptides are displayed on class I major histocompatibility complex (MHC) of the tumor cells. A handful of tumor-associated antigens have been identified and many of them are weak antigens. As an alternative strategy, strongly antigenic foreign peptides are delivered to the tumor, marking them for CTL recognition. To establish the principle of this new strategy, in vitro and in vivo tumor destruction was tested with BALB/c CTL to L<sup>d</sup>-associated beta-galactosidase ( $\beta$ -gal) peptide p876. In vitro, anti-p876 CTL destroyed tumor cells in a single-cell suspension or in 3-D tumor boluses when exogenous p876 was added. Exogenous IL-2 was required to sustain CTL activity for complete destruction of tumor boluses. In vivo, BALB/c mice were immunized with p876 and a CD4 activating Pan DR reactive epitope (PADRE). PADRE, which binds to several different MHC class II antigen and activates CD4 T cells, induced delayed-type hypersensitivity and stimulated T cell proliferation. Immunized mice were injected with tumor cells loaded with p876 and mixed with PADRE. Starting from the day after tumor injection, mice received five rounds of peptide injection at the tumor sites and all tumors were rejected. Injection with saline had no effect. Injection with PADRE had minor anti-tumor activity. Immunization and treatment with p876 alone was not protective. Therefore, by delivering CD4 and CD8 reactive foreign peptides to the tumor, peptide-specific T cells rejected the tumors as demonstrated by the in vitro and in vivo tests. © 2001 Elsevier Science B.V. All rights reserved.

**Keywords:** CTL; Antigenic peptide; Cancer therapy; Prodrug

### 1. Introduction

Vaccination with tumor-associated, major histocompatibility complex (MHC) class I antigen-re-

stricted immunogenic peptides can induce tumor antigen-specific immunity. BALB/c mammary tumor growth was inhibited in mice immunized with peptide E474 from the envelope protein of mouse mammary tumor virus (MMTV) (Wei et al., 1996a,b). HLA-A1-associated MAGE-3 peptide induced tumor shrinkage in three of sixteen melanoma patients (Marchand et al., 1995). Immunization with HLA-A2-restricted gp100 peptide combined with systemic IL-2 treatment induced partial response in 42% of melanoma patients (Rosenberg et al., 1998). Patients with metastatic melanoma showed complete

*Abbreviations:* CTL, cytotoxic T cells; MHC, major histocompatibility complex; CFA, complete Freund's adjuvant; MMTV, mouse mammary tumor virus; PADRE, Pan DR reactive epitope;  $\beta$ -Gal, beta-galactosidase.

\* Corresponding author. Tel.: +1-313-833-0715x2360; fax: +1-313-831-7518.

E-mail address: weiw@karmanos.org (W.-Z. Wei).

response after they were immunized with dendritic cells loaded with three melanoma peptides (Nestle et al., 1998). These examples indicated the anti-tumor efficacy of peptide-specific cytotoxic T cells (CTL).

Several limitations in tumor-specific immunotherapy prompted us to search for alternative approaches. Antigen expression by tumor cells is heterogeneous and T cells recognizing tumor-specific antigens may eliminate antigen positive tumor cells, but allow the expansion of antigen negative cells. A limited number of tumor-associated antigens have been identified and many of them are weak antigens (Pardoll, 1998). When immunity to tumor-associated self antigens is successfully induced, autoimmunity becomes a risk factor. An alternative approach to exploit effector T cells without being limited by the tumor-associated antigens will be of great advantage.

Chemotherapeutic agents can be delivered to the tumors under the cover of pro-drugs. A nontoxic glucuronyl-spacer-doxorubicin prodrug HMR 1826 was activated selectively by  $\beta$ -glucuronidase which is found at high concentration at the tumor site (Murdter et al., 1997; Bosslet et al., 1998). Treatment with HMR 1826 resulted in tumor destruction without systemic toxicity. The findings with HMR 1826 encouraged the strategy of delivering antigenic peptides under the cover of pro-peptides. A pro-peptide can be synthesized by conjugating glucuronide to the amino terminus through the self-immolative linker (Rawale et al., 2001). The pro-peptide does not bind to MHC class I antigen, but  $\beta$ -glucuronidase can cleave and release the active peptide, which then binds to MHC class I antigen. The current study is undertaken to establish the in vitro and in vivo assay methods for measuring anti-tumor activity of CTL to foreign peptides.

Therapeutic potential of CTL against a solid tumor was first demonstrated in vitro in collagen gel (Wei et al., 1985, 1996a,b). Tumor cells grew as a 3-D mass with intracellular junctions and resembled a solid tumor. The tumor mass could be maintained in the collagen gel for 1–2 weeks, providing prolonged observation period and a realistic in vitro system to measure anti-tumor activity (Gavin et al., 1993). Activated T cells migrated through the collagen gel (Ratner et al., 1992) and inhibition of tumor growth was monitored by daily measurement of the tumor size.

To sustain CTL activity in vivo, CD4 T cells were activated by Pan DR reactive epitope (PADRE) aK(X)VAAWTLKAAa (a is D-alanine and X is cyclohexylalanine), a synthetic peptide containing amino acid sequences derived from the circumsporozoite protein (CSP) from *Plasmodium falciparum*. PADRE binds multiple LHA-DR allospecificity and murine I-A<sup>b/d</sup> or I-E<sup>b/d</sup> antigens (Alexander et al., 1994) and induces helper T cell activity. The efficacy of PADRE to enhance anti-tumor CTL activity was examined.

## 2. Materials and methods

### 2.1. Mice and cell lines

BALB/c mice originally obtained from the Cancer Research Laboratory, Berkeley, CA were bred by brother–sister mating in our animal care facility. The use and care of experimental mice are in accordance with the guidelines of Wayne State University Animal Investigation Committee. Mouse mammary tumor cell line D2F2 was cloned from a spontaneous mammary tumor, which arose in a BALB/c hyperplastic alveolar nodule line D2 (Mahoney et al., 1985). MMT cell line 168 was cloned from a spontaneous tumor in a BALB/cfC3H mouse which was the offspring of BALB/c mice foster nursed on C3H/HeJ mothers infected with MMTV(C3H) (Wei et al., 1993). Cell line 293 (American Type Culture Collection, Manassas, VA) is a human embryonic kidney cell line transformed with Ad5 E1A and E1B genes, and supports the propagation of E1 deleted recombinant adenovirus. All tissue culture reagents were obtained from GIBCO Laboratories (Grand Island, NY) unless otherwise specified. The cell lines were maintained in Dulbecco's modified Eagle's medium (DME) supplemented with 5% heat-inactivated fetal calf serum (HyClone, Logan, UT), 10% NCTC 109 medium (Sigma, St. Louis, MO), 8  $\mu$ g/ml bovine crystalline insulin (Sigma), 1 mM oxalacetic acid, 0.5 mM sodium pyruvate, 2 mM L-glutamate, 0.1 mM MEM nonessential amino acids, 100 units/ml penicillin and 100  $\mu$ g/ml streptomycin.

## 2.2. Peptides

Beta-galactosidase ( $\beta$ -gal) peptide p876 TPH-PARIGL and PADRE aK(X)VAAWTLKAAa (a is D-alanine and X is cyclohexylalanine) were synthesized at Genemed Synthesis (South San Francisco, CA).

## 2.3. Establishment of peptide-specific CTL

BALB/c mice were injected s.c. at the tail base with 100  $\mu$ g of p876 emulsified in 0.1 ml of 50% complete Freund's adjuvant (CFA). At 10 days after the injection, the spleen and lymph nodes were aseptically removed from immunized mice and a single-cell suspension was prepared. The freshly isolated cells were cultured with peptide-loaded, irradiated splenocytes in 12-well plates in RPMI 1640 medium containing 50  $\mu$ M of 2-mercaptoethanol, 10% fetal calf serum, 5% mixed leukocyte culture supernatant and 18 IU/ml of recombinant hIL-2 (Cetus, Emeryville, CA). The ratio of stimulators to responders was 2:1.

To prepare the stimulators, autologous splenocytes were incubated with 100  $\mu$ g/ml of p876 for 2 h, exposed to 1000 rad irradiation from a Cesium irradiator and washed to remove excess peptides before they were added to the culture. The cultured cells were re-stimulated with peptide-loaded splenocytes every 7–10 days.

Mixed leukocyte culture was prepared by incubating BALB/c splenocytes with irradiated human peripheral blood leukocytes for 48 h in RPMI 1640 medium with 10% FCS. The ratio of mouse and human cells was 1:1 and the total cell concentration was  $5 \times 10^6$  cells/ml.

## 2.4. $^{51}\text{Cr}$ release assay

D2F2 tumor target cells were labeled by incubating  $10 \times 10^6$  cells with 100  $\mu$  Ci  $\text{Na}^{51}\text{CrO}_4$  (NEN Research Products, Boston, MA) in 1 ml of complete DME at 37 °C for 2 h. In some experiments, the target cells were loaded with p876 peptide by adding 100  $\mu$ g of p876 during the incubation. The unincorporated  $^{51}\text{Cr}$  and unbound peptide was removed by three washes with Hanks' balanced salt solution containing 2% calf serum and 2 mM Hepes buffer.

Graded numbers of effector cells were mixed with  $1 \times 10^5$  labeled target cells in 200  $\mu$ l of DME in the wells of round bottom microtiter plates. After centrifugation at  $200 \times g$  for 1 min, the plate was incubated at 37 °C for 4.5 h. After the incubation, the plate was centrifuged at  $480 \times g$  for 10 min and a 100- $\mu$ l aliquot was removed from each well for counting in the gamma counter. The percent lysis was calculated as follows

$$\% \text{ specific lysis} = 100 \times (\text{cpm}_{\text{test}} - \text{cpm}_{\text{medium}}) / (\text{cpm}_{\text{max}} - \text{cpm}_{\text{medium}}).$$

The  $\text{cpm}_{\text{max}}$  was determined by adding 1/6 N HCL to wells containing  $^{51}\text{Cr}$ -labeled target cells. Each group contained four replicates.

## 2.5. A 3-D collagen gel assay

The growth and lysis of tumor cell boluses in the collagen were previously reported (Wei et al., 1996a,b) and are briefly described here. Collagen stock solution was prepared from rat tail tendons. The complete collagen mixture contained 12 parts of cold collagen stock, 2 parts of  $7.5 \times \text{RPMI 1640 medium}$ , 0.6 parts of 7.5% sodium bicarbonate and 0.5 parts of 0.34 N NaOH. This mixture stayed in liquid form if kept on ice. Tumor cell boluses were prepared by suspending  $1 \times 10^5$  tumor cells with or without CTL in 1  $\mu$ l of complete collagen mixture and embedded between two layers of collagen gels. The gel was bathed in RPMI 1640 medium supplemented with 50  $\mu$ M of 2-mercaptoethanol, 10% fetal calf serum, 5% mixed leukocyte culture supernatant and 18 IU/ml of recombinant hIL-2 (Cetus). Tumor cells established inter-cellular junction and formed a cell mass during overnight culture (Wei et al., 1985, 1996a,b). In some experiments, the CTL were added to the top of the collagen and allowed to migrate into the gel. Peptide p876 was added the day after CTL were embedded or added. The plates were incubated at 37 °C and tumor growth was measured every other day.

Tumor cell boluses embedded in collagen gel were examined with an inverted microscope equipped with a split image tracing device, using  $1 \times$  objective. The projected image of the cell bolus

was traced and the surface area was calculated. The result was expressed as the square root of the measured area, which represented the mean diameter of the bolus, multiplied by a constant. The difference between the test and control group was determined with one-tail Student's *t*-test. There were four replicates in each group.

### 2.6. Foot pad swelling test

BALB/c female mice were immunized by s.c. injection with 20 µg of PADRE and 100 µg of p876 in 50% IFA, and challenged 2 weeks later in the foot pads with either or both peptides in 20 µl of saline. The thickness of the feet was measured with a caliper at 24 and 48 h after injection. The significance of the swelling was determined by comparing each test group with the saline control group using Student's *t*-test. There were seven to eight mice in each group.

### 2.7. Lymphocyte proliferation assay

Lymph nodes were removed aseptically from mice, and a single-cell suspension was prepared in RPMI 1640 medium supplemented with 4% fetal calf serum. Suspended cells were distributed to round bottom wells of a 96-well plate and each well received  $1.5 \times 10^5$  cells. Test peptide at varying concentrations or Concanavalin A (con A) at 1 µg/ml was added to each well. There were four replicates in each group. Cells were incubated in 5% CO<sub>2</sub> at 37 °C for 4 days. Each well received 1 µCi of <sup>3</sup>H-thymidine, and the cells were further incubated for 4 h before they were harvested with Harvester96 (Tomtec, Hamden, CT), and the incorporated <sup>3</sup>H was counted with TRILUX liquid scintillation and luminescence counter (Wallac, Turku, Finland).

### 2.8. Preparation of AdCMVLacZ

The construction of AdCMVLacZ was previously reported (Acsadi et al., 1994). The recombinant virus was purified through two rounds of plaque purification, propagated in 293 cells, and isolated on a discontinuous cesium chloride gradient. The virus was collected and the salt was removed with a Se-

phadex column. The quantity of viral particles was estimated by measuring the optical density at 260 nm and the plaque-forming unit (PFU) was determined by direct plaque assay with 293 cells.

### 2.9. Tumor growth inhibition assay

Female BALB/c mice at 6 weeks of age were immunized by intracutaneous injection with 0.1 ml of saline containing  $1 \times 10^{10}$  PFU of AdCMVLacZ, and boosted, 2 weeks later, by s.c. injection at the tail base with 0.1 ml of 50% IFA containing 100 µg of p876 and/or 20 µg of PADRE. At 2 weeks after vaccination, mice were injected s.c. on the flank with  $1 \times 10^5$  untreated or peptide-coated D2F2 cells. Starting the day after tumor cell challenge, mice received subcutaneously, every other day, at the tumor site, 0.1 ml of saline containing 100 µg of p876 and/or 40 µg PADRE. A total of five injections were administered and tumor growth was monitored weekly. There were eight mice in each group.

## 3. Results

### 3.1. Inhibition of tumor growth by p876-specific CTL *in vitro*

BALB/c CTL to β-gal peptide p876 was established as described in Materials and methods. After the fifth *in vitro* stimulation, more than 99% of the cells were CD8+ (not shown). CTL were frozen between the seventh and tenth stimulation and they were readily reactivated. The specificity was measured by the <sup>51</sup>Cr release assay. CTL lysed peptide p876-coated BALB/c mouse mammary tumor D2F2 cells, but not untreated D2F2 cells (Fig. 1). Expression of L<sup>d</sup> by D2F2 cells was verified by flow cytometry (not shown).

The activity of p876-specific CTL against a solid tumor was tested in a 3-D collagen gel to access peptide loading and CTL lysis of a tumor mass. A total of  $1 \times 10^5$  D2F2 cells and  $3 \times 10^5$  CTL were embedded together in 1 µl of collagen (Fig. 1B). After an overnight incubation when the cell bolus was established, p876 at 5 or 20 µg/ml was added to the culture and tumor growth was monitored for 7

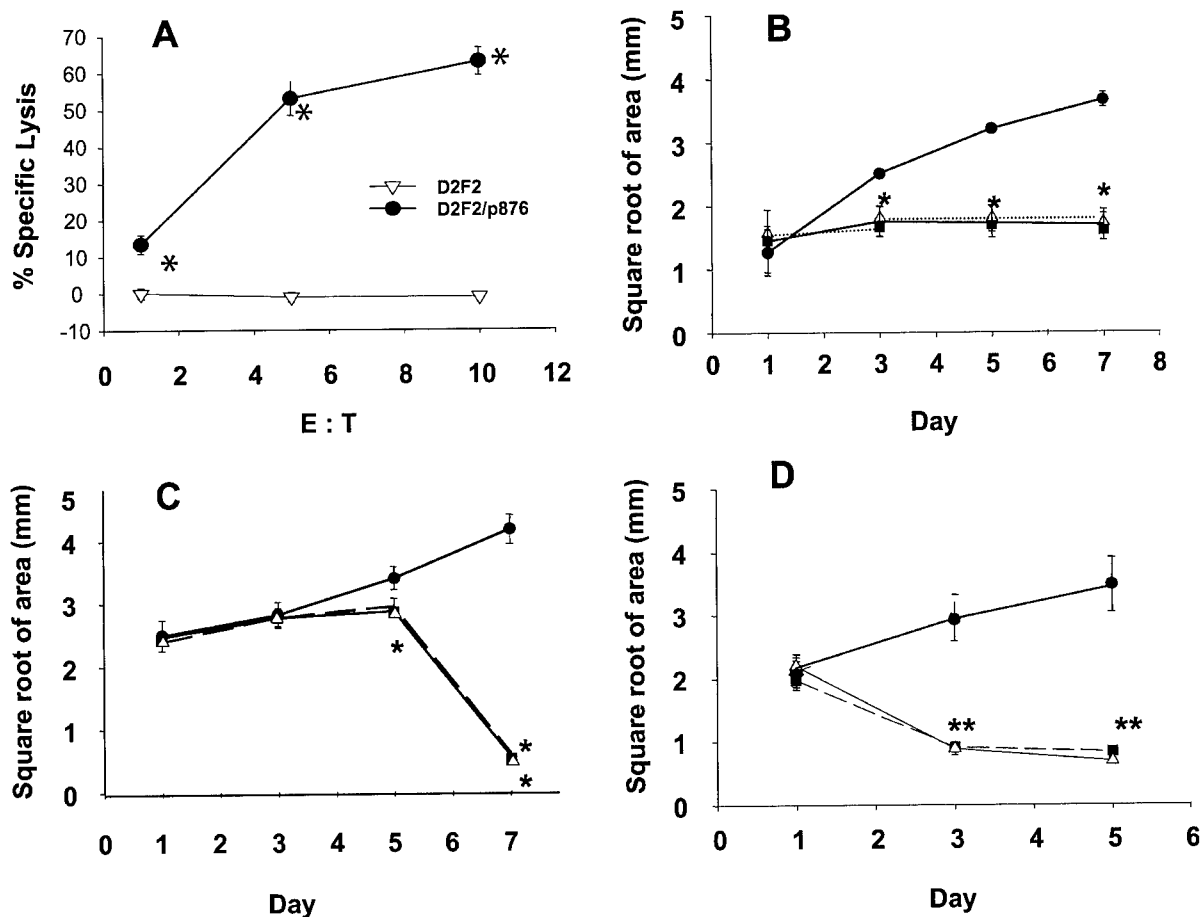


Fig. 1. Anti-tumor activity of anti-p876 CTL. (A) Cytotoxic activity of anti-p876 CTL measured by the  $^{51}\text{Cr}$  release assay. The D2F2 cells were labeled with  $\text{Na}^{51}\text{CrO}_4$  in the presence (solid circle) or absence (open triangle) of p876 and chromium release was measured after a 4.5-h incubation at  $37^\circ\text{C}$  with anti-p876 CTL. There were four replicates in each group. (B–D) Inhibition of D2F2 tumor growth by anti-p876 CTL in 3-D collagen gel. (B) A total of  $1 \times 10^5$  D2F2 cells were co-embedded with  $3 \times 10^5$  anti-p876 CTL in  $1 \mu\text{l}$  of collagen. After an overnight incubation at  $37^\circ\text{C}$ , p876 at 5 (solid square) or 20 (open triangle)  $\mu\text{g}/\text{ml}$  was added to the culture. Control group (solid circle) did not receive peptide. Alternatively,  $1 \times 10^5$  D2F2 (C) or 168 (D) cells were embedded alone in  $1 \mu\text{l}$  of collagen, and  $3 \times 10^5$  anti-p876 CTL were added to the collagen gel after a tumor mass is established overnight. CTL migrated through the gel and p876 peptide was added after 24 h. Tumor growth was measured by the square root of the growth area. \* indicates significant difference between the control and test groups at  $P < 0.005$ .

days. Without exogenous p876, D2F2 cells, co-embedded with anti-p876 CTL, grew progressively. The addition of p876 resulted in profound inhibition of tumor growth. Therefore, tumor cells in the solid mass were loaded with the exogenous peptide and are targets of CTL lysis.

Anti-tumor activity was further tested by embedding D2F2 cells in the collagen to establish a tumor mass before CTL and peptides were added. Therefore,  $1 \times 10^5$  D2F2 cells were embedded and incu-

bated overnight to establish a tumor mass with intercellular junctions (Miller et al., 1985) (Fig. 1C). CTL were added to the top of the gel and approximately 20–40% of CTL migrated through the collagen in 18 h (Ratner et al., 1992) when peptide p876 at 5 or 20  $\mu\text{g}/\text{ml}$  was added. Complete disintegration of the tumor boluses was observed after 5 days of culture. Vigorous proliferation of CTL was observed in and around the tumor (not shown). The destruction of tumor cell boluses was verified with another mouse

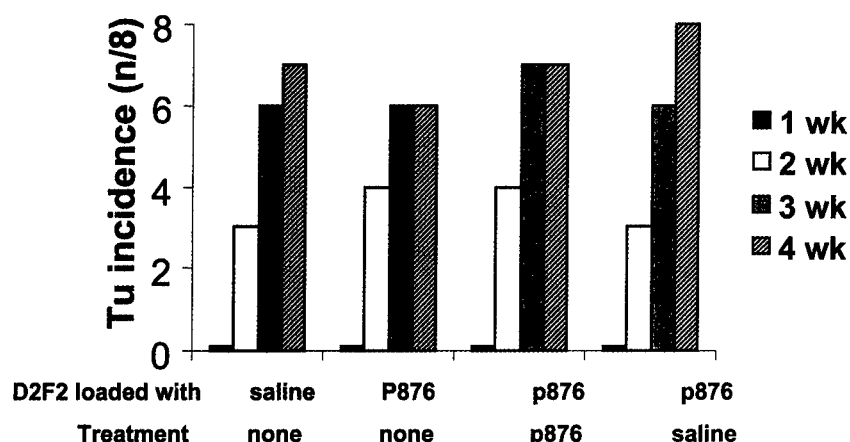


Fig. 2. D2F2 tumor growth in p876-immunized and treated mice. BALB/c mice were immunized i.c. with  $1 \times 10^{10}$  PFU of *AdCMVLacZ* and boosted with  $\beta$ -gal p876. At 2 weeks after vaccination, each mouse received s.c.,  $1 \times 10^5$  D2F2 cells coated or not-coated with  $\beta$ -gal p876. Mice injected with peptide-coated cells received peri-lesional injection of 200  $\mu$ g p876 in 100  $\mu$ l of saline every other day, five times, starting the day after tumor cell injection. Control mice received saline injection or were not treated.

mammary tumor cell line 168, which also expressed L<sup>d</sup> antigen (Fig. 1D). The extended culture period of the tumor boluses will be a particular advantage for testing pro-peptides, which are released by tumor-associated enzymes.

### 3.2. Effect of p876 on tumor growth in BALB/c mice

To test anti-tumor activity of p876-specific CTL in vivo, BALB/c mice were immunized with *Ad-CMVlacZ* to activate  $\beta$ -gal specific CTL and boosted in 2 weeks with 100  $\mu$ g p876 in IFA. At 2 weeks after peptide immunization, mice were challenged with D2F2 cells pre-coated with p876. Starting the day after tumor cell challenge, mice were injected at the tumor site, every other day, with 200  $\mu$ g of p876 in 0.1 ml saline. There were five injections in 10

days. Control mice received saline or were not treated. One group received uncoated D2F2 cells. There was no demonstrable protection in any of the treatment group (Fig. 2). We previously showed that immunization with irrelevant peptides had no effect on tumor growth (Wei et al., 1996a,b). The inability to inhibit tumor growth by local administration of p876 may be due to inadequate number of CTL at the tumor site as a result of inadequate cytokine or accessory signals. In the 3-D collagen, exogenous IL-2 was required to achieve complete tumor destruction, indicating that continued stimulation and expansion of the CTL was necessary for controlling tumor growth (Wei et al., 1996a,b).

### 3.3. T cell activation by PADRE

To enhance local recruitment or maintenance of CTL, CD4 T cell reactive peptide was incorporated

Table 1  
Delayed-type hypersensitivity induced by PADRE

Vaccination	Challenge	Change in foot pad thickness (mm)			
		Day 1	P value	Day 2	P value*
PADRE/IFA <sup>a</sup>	PADRE	57.4 $\pm$ 22.0	< 0.0002	36.1 $\pm$ 19.3	< 0.002
IFA	PADRE	13.4 $\pm$ 19.3	> 0.05	14.0 $\pm$ 13.9	> 0.05
PADRE/IFA	Saline	2.9 $\pm$ 7.4	> 0.05	6.0 $\pm$ 18.4	> 0.05
IFA	Saline	6.6 $\pm$ 16.0	> 0.05	3.6 $\pm$ 6.6	> 0.05

<sup>a</sup>Mice were immunized and challenged as described in Materials and methods. There were eight mice in each group.

\* P value was determined by the Student's *t*-test.

Table 2  
Delayed-type hypersensitivity induced by PADRE and  $\beta$ -gal

Challenge	Change in foot pad thickness (mm)			
	Day 1	<i>P</i> value	Day 2	<i>P</i> value*
PADRE	38.6 $\pm$ 6.6	< 0.001	24.3 $\pm$ 8.6	< 0.01
$\beta$ -gal/ PADRE	49.3 $\pm$ 25.4	< 0.05	39.4 $\pm$ 18.4	< 0.0005
$\beta$ -gal	12.1 $\pm$ 14.7	> 0.05	8.7 $\pm$ 14.7	> 0.05
Saline	3.9 $\pm$ 7.1		5.3 $\pm$ 9.6	

Mice were immunized and challenged as described in Materials and methods. There were eight mice in each group.

\* *P* value was determined by the Student's *t*-test.

into the treatment regimen. PADRE, which binds to a wide range of human and murine MHC class II antigen, was tested as local CD4 T cell activator. The immunogenicity of PADRE was tested by the foot pad swelling assay. BALB/c mice were immunized s.c. with 20  $\mu$ g of PADRE in 0.1 ml of 50% CFA and challenged 2 weeks later in the foot pad with 40  $\mu$ g of PADRE in 20  $\mu$ l of saline. Significant swelling was induced by PADRE both 1 and 2 days after challenge (Table 1). Injection of PADRE in control mice or injection of saline in PADRE-immunized mice did not induce significant swelling. Therefore, PADRE-specific T cells were activated in vivo by peptide immunization. Delayed-type hypersensitivity, induced by a combination of p876 and PADRE, was also tested. Mice were immunized with 100  $\mu$ g of p876 and 20  $\mu$ g of PADRE in Incomplete Freund's adjuvant and challenged 2 weeks later with p876, PADRE or a mixture of both peptides. Significant increase in foot pad thickness was observed

after PADRE was injected (Table 2). Injection of both p876 and PADRE further enhanced the immune reactivity. p876 alone did not have significant effect. These results support the immunogenicity of PADRE in BALB/c mice and an enhanced inflammatory reaction when both MHC class I and II antigen reactive peptides were present.

Activation of T cells was also measured by lymphocyte proliferation assay (Table 3). Lymph node cells were prepared from BALB/c mice 2 weeks after mice were immunized s.c. with 20  $\mu$ g of PADRE in 0.1 ml of 50% CFA. Immune cells were incubated with 0.1–10  $\mu$ g of PADRE and  $^3$ H-thymidine incorporation was measured after 4 days of culture. Incorporation of  $^3$ H-thymidine was increased by more than 100 fold in sensitized lymphocytes when incubated with 10  $\mu$ g of PADRE. Naïve lymphocytes did not demonstrate significant activity, supporting specific immune activation by PADRE.

### 3.4. Anti-tumor activity of p876 and PADRE

To test the therapeutic effect of p876 and PADRE, BALB/c mice were immunized with AdCMVLacZ and boosted in 2 weeks with 100  $\mu$ g p876 and 20  $\mu$ g PADRE in IFA. At 2 weeks after the second immunization, mice received s.c. D2F2 cells pre-coated with p876 and mixed with PADRE. Mice were treated five times at the tumor site with a mixture of PADRE and p876, every other day. Complete inhibition of D2F2 tumor growth was observed in mice treated with PADRE and p876 (Fig. 3). Immunization and treatment with PADRE alone had a minor anti-tumor activity (not shown). These re-

Table 3  
Lymphocyte proliferation induced by PADRE

Treatment <sup>a</sup>	Mean $\pm$ S.D. (cpm)			
	Immune	<i>P</i> value	Normal	<i>P</i> value*
Medium	145 $\pm$ 141		47 $\pm$ 14	
PADRE 0.1 $\mu$ g/ml	269 $\pm$ 136	> 0.05	40 $\pm$ 10	> 0.05
PADRE 5 $\mu$ g/ml	6360 $\pm$ 974	< 0.005	183 $\pm$ 117	> 0.05
PADRE 10 $\mu$ g/ml	18 167 $\pm$ 3784	< 0.005	137 $\pm$ 158	> 0.05
Con A 1 $\mu$ g/ml	92 765 $\pm$ 11 148	< 0.005	78 263 $\pm$ 16 138	< 0.005

<sup>a</sup> Lymph node cells were pooled from three mice 2 weeks after PADRE immunization and stimulated with PADRE as described in Materials and methods.

\* *P* value was determined by the Student's *t*-test.

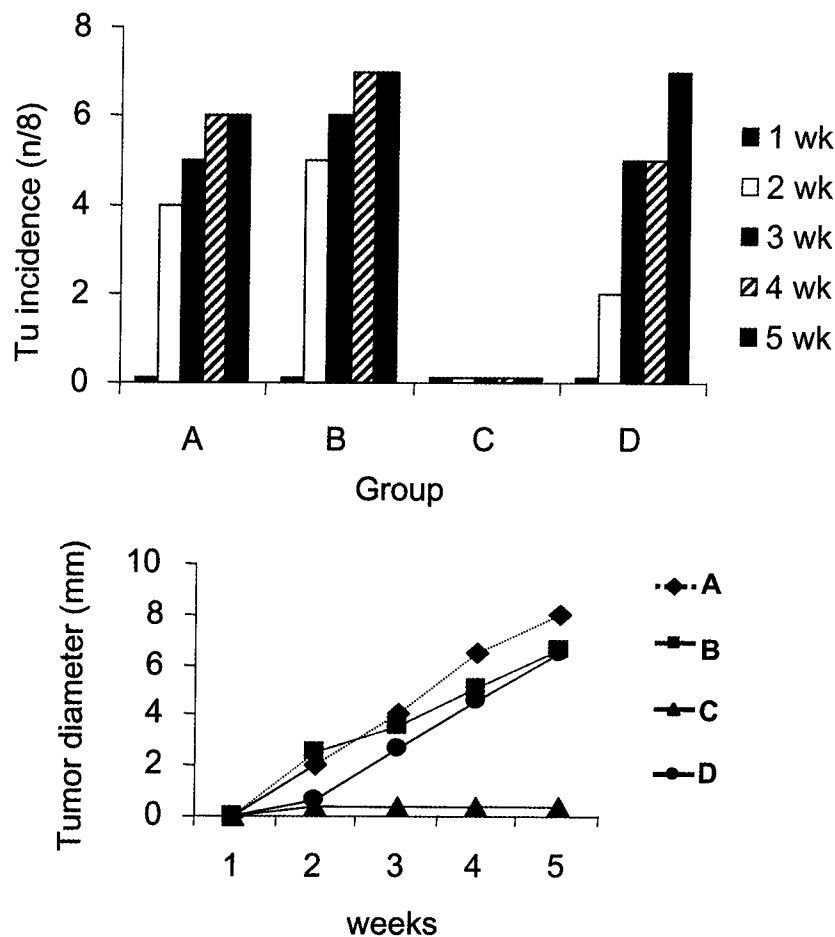


Fig. 3. Inhibition of D2F2 tumor growth by PADRE and p876. BALB/c mice were immunized i.c. with  $1 \times 10^{10}$  PFU of *AdCMVLacZ* and boosted 2 weeks later by s.c. injection with 100  $\mu$ g of p876 and 20  $\mu$ g of PADRE in 50% IFA. At 2 weeks after vaccination, each mouse received s.c.  $1 \times 10^5$  D2F2 cells (A) or D2F2 cells coated with  $\beta$ -gal p876 and mixed with PADRE (B–D). Mice in group (C) received a peri-lesional injection with p876 (200  $\mu$ g) and PADRE (40  $\mu$ g) in 100  $\mu$ l of saline every other day, five times, starting from the day after tumor cell injection. Control mice were not treated (B) or received saline injections (D). The lower panel showed the average tumor diameter in groups A–D.

sults demonstrated that activation of both CD4 and CD8 T cells at the tumor site with foreign peptide eliminated tumors loaded with the specific peptides.

#### 4. Discussion

This study outlines a scheme for inducing and measuring anti-tumor activity mediated by T cells to foreign peptides. CTL to  $\beta$ -gal peptide p876 eliminated tumor boluses in a 3-D matrix, demonstrating

effective loading of p876 in a solid mass, migration of CTL to the tumor and sustained CTL killing at the tumor site. Exogenous IL-2 was necessary to maintain CTL activity. In vivo, tumors were rejected if they were loaded with p876 and PADRE and treated locally with the same peptides. PADRE was required to sustain CTL activity in vivo. Although tumor cells were loaded with peptides in this study, it should be possible to deliver foreign peptides onto tumor cells in vivo by appropriate vehicles. The pro-peptide developed by our group does not bind MHC class I



antigen until the active peptide is released by beta-glucuronidase (Rawale et al., 2001 ID: 2226). Based on the same principle, doxorubicin prodrug HMR 1826 was activated selectively at the tumor site by  $\beta$ -glucuronidase and demonstrated anti-tumor activity without systemic toxicity (Murdter et al., 1997; Bosslet et al., 1998). It is anticipated that pro-peptide can be delivered to the tumors by systemic administration. The 3-D tumor growth assay and in vivo tumor inhibition in the presence of PADRE will be useful for testing pro-peptides and other derivatives of MHC class I antigen-associated peptides.

$\beta$ -gal encoded by *Escherichia coli lacZ* gene is widely used as a genetic tracer. It is immunogenic and L<sup>d</sup>-restricted epitope p876 TPHPARIGL was previously defined (Rammensee et al., 1989; Gavin et al., 1993). Immunization of BALB/c mice with p876 inhibited the growth of a colon carcinoma genetically engineered to express  $\beta$ -gal, supporting the cytotoxic activity of anti- $\beta$ -gal CTL (Specht et al., 1997). Here, we showed that p876 effectively marked a solid tumor when loaded exogenously onto the tumor cells.

Tumor growth in the 3-D collagen gel is a useful indicator of CTL activity against solid tumors. When CTL were co-embedded with the tumor cells, tumor growth was inhibited, but some tumor cells remained after 2 weeks (not shown). When the same number of CTL were added exogenously, less than half migrated through the gel. These migratory T cells caused complete disintegration of the tumor. When CTL were packed in close proximity in the cell bolus, they may recognize neighboring CTL loaded with p876 and lyse each other. Alternatively, tumor cells may demonstrate immune suppressive activity, e.g. Fas ligand on tumor cells may induce T cell apoptosis. (Whiteside and Rabinowich, 1998; Sarma et al., 1992; Zeytun et al., 1997). These mechanisms can significantly reduce the number of functional effectors in the tumor mass. T cells present in human tumors may be similarly ineffective because of the hostile microenvironment. When the hosts are immunized with foreign peptides, peptide-specific T cells will reside in the peripheral lymphoid organs until they are recruited to the tumor site by the injected peptides. When recruited to the tumor from the periphery, they may be more efficacious than resident T cells against tumor cells.

The in vivo therapeutic effect was striking when both CD4 and CD8 reactive peptides were delivered to the tumor and all tumors were rejected. Delivery of p876 alone was not effective, indicating the need to recruit and sustain CTL activity in vivo. Treatment with PADRE alone had a minor anti-tumor effect (not shown), but was not nearly as effective as p876 and PADRE together. PADRE can bind to I-A<sup>d</sup> or I-E<sup>d</sup> antigen expressed by the professional antigen presenting cells (APC) or non-professional APC like the endothelial cells (Rose, 1997) and activate CD4 T cells. Interaction between activated CD4 T cells and APC can further enhance the antigen presenting capacity of the latter (Bennett et al., 1998; Schoenberger et al., 1998). Cytokines generated by activated CD4 T cells recruit and activate additional APC, CD8 T cells and non-specific inflammatory cells.

With the proposed scheme, several major obstacles in tumor immunotherapy, namely, tolerance to self-antigens, induction of autoimmunity or the lack of tumor-specific antigens may be circumvented. Because the peptide is foreign, tolerance to the antigen is not an issue. After the peptide treatment is terminated, there is no lingering immune reactivity to self-antigens. Importantly, this therapy does not rely on the expression of tumor-specific antigens because the therapeutic peptides are administered exogenously. Several peptides can be administered simultaneously or sequentially to enhance the therapeutic effect. Each round of peptide treatment represents a booster shot and can increase the frequency of reactive T cells. The observation that 1-day tumor can be eliminated by local delivery of foreign peptides suggests that this strategy may be useful as an adjuvant therapy to eliminate residual tumors at the surgical sites or small tumors not accessible by surgery.

In summary, excellent therapeutic effect was achieved by local delivery of foreign peptides to the tumors. This demonstration provides the basis for developing new strategies to deliver therapeutic foreign peptides to the tumor.

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**Synthesis and Biological Activity of the  
Prodrug of Class I Major  
Histocompatibility Peptide GILGFVFTL  
Activated by  $\beta$ -Glucuronidase**

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**Sharad Rawale, Lew M. Hrihorczuk, Wei-Zen Wei, and  
Jiri Zemlicka**

Barbara Ann Karmanos Cancer Institute, Wayne State University  
School of Medicine, Detroit, Michigan 48201-1379, and Central  
Instrumentation Facility, Department of Chemistry, Wayne State  
University, Detroit, Michigan 48202

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# Synthesis and Biological Activity of the Prodrug of Class I Major Histocompatibility Peptide GILGFVFTL Activated by $\beta$ -Glucuronidase<sup>1</sup>

Sharad Rawale,<sup>†</sup> Lew M. Hrihorczuk,<sup>‡</sup> Wei-Zen Wei,<sup>†</sup> and Jiri Zemlicka<sup>\*,†</sup>

Barbara Ann Karmanos Cancer Institute, Wayne State University School of Medicine, Detroit, Michigan 48201-1379, and Central Instrumentation Facility, Department of Chemistry, Wayne State University, Detroit, Michigan 48202

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The first synthesis of a prodrug of HLA-A2.1 associated antigenic influenza peptide 2a was accomplished. Two methods for synthesis of prodrugs of antigenic peptides activated by  $\beta$ -glucuronidase and comprising a self-immolative 3-nitrobenzyloxycarbonyl moiety were investigated. Reaction of  $\beta$ -glucuronic acid glycoside of 4-hydroxy-3-nitrobenzyl alcohol (3) with *N,N'*-disuccinimidyl carbonate (DSC) followed by conjugation with AlaOMe, Gly, Thr, Phe-Leu, and Leu-Arg gave carbamates 4a–4f. Deacetylation of 4b and 4e with MeONa/MeOH gave  $\beta$ -glucuronides 5b and 5e. Compound 5e was converted to  $\beta$ -glucuronic acid conjugate 6e by the action of pig liver esterase (PLE). Compound 6e is a substrate for  $\beta$ -glucuronidase. Method of a direct introduction of the prodrug residue into antigenic nonapeptide GILGFVFTL (2b) failed. Alternately, glycine conjugate 5b was activated to pentafluorophenyl ester 10. Model coupling of 10 with Phe-Leu gave tripeptide conjugate ester 11a which was hydrolyzed by PLE to uronic acid 12. Condensation of 10 with octapeptide ILGFVFTL (9) gave prodrug precursor 11b. Octapeptide 9 was prepared by de novo synthesis using a racemization-free fragment coupling method. Ester hydrolysis with Ba(OH)<sub>2</sub>/MeOH gave the target prodrug 2a which is a substrate for  $\beta$ -glucuronidase. Prodrug 2a does not bind to HLA-A2.1 of T2 human cells defective in major histocompatibility complex I (MHC I)-associated peptide processing. Addition of  $\beta$ -glucuronidase restored the binding to the level observed with parent nonapeptide 2b although higher concentrations of prodrug 2a and enzyme were necessary.

In an effort to design more effective and less toxic drugs against cancer, attention has turned in recent years to prodrugs of antitumor agents. Most of these approaches rely on specific enzymes capable of converting the prodrugs into active species at a tumor site. Selective drug release should reduce the side effects associated with other forms of chemotherapy. This is also the principle of antibody- and gene-directed prodrug therapy (ADEPT<sup>2,3</sup> and GDEPT<sup>4</sup>). Prodrugs comprising a carbohydrate moiety amenable to enzyme action attached through a self-immolative benzyloxycarbonyl linker to an anticancer drug (e.g., doxorubicin or daunorubicin, compounds 1a–1c, Chart 1) have attracted a considerable interest.<sup>5–9</sup> The prodrug-activating enzymes include  $\beta$ -glucuronidase and  $\alpha$ - or  $\beta$ -galactosidase. Prodrug 1b ( $R_2$  = NO<sub>2</sub>, HMR 1826) activated by  $\beta$ -glucuronidase liberated in necrotic tumors<sup>10</sup> has an increased efficacy and reduced toxicity relative to that of the parent drug.<sup>11</sup> It is a promising candidate for clinical studies.<sup>12</sup>

We have reasoned that this prodrug concept can be extended to antigenic oligopeptides<sup>13</sup> (8–10 a.a.) which bind to an extracellular domain of class I major histocompatibility complex (MHC I) or human leukocyte antigen (HLA) and are recognized by CD8 positive cytotoxic T lymphocytes (CTL). Interaction between T

cell receptor (TCR) and class I HLA/peptide complex on the target cells triggers CTL activity which mediates target cell lysis.<sup>14</sup> The HLA I peptides on tumor cells originate from endogenous proteins but they can be replaced exogenously by synthetic peptides. It has been shown that CTL can kill tumor cells very effectively if the latter were loaded with appropriate peptides.<sup>15</sup> Thus, mice immunized with tumor-associated peptides rejected tumors expressing the specific peptides.

We can then assume that tumors can be rejected if any antigenic peptide which binds to the appropriate MHC is delivered to the tumor in an immunized host. Systemic delivery of such antigenic peptide is not possible because it will bind to all nucleated cells resulting in severe toxicity. Effective antitumor activity may be achieved if the peptide is converted to a suitable prodrug which will release the peptide only at the tumor site. Glucuronic acid linked through a benzyloxycarbonyl spacer to the N-terminus of the antigenic peptide seems to fulfill such requirements. The anchor residues at or near the N- and C-termini of peptides are necessary for effective interaction<sup>16</sup> with HLA or MHC I. Blocking one of these sites by a bulky prodrug grouping should eliminate or diminish such a binding. As indicated above for prodrugs 1a–c,  $\beta$ -glucuronidase in the microenvironment of tumors can effectively generate an active drug. Substitution at the N-terminus should also reduce the susceptibility of the prodrug toward enzymatic degradation (peptidases) in cell culture and in vivo.

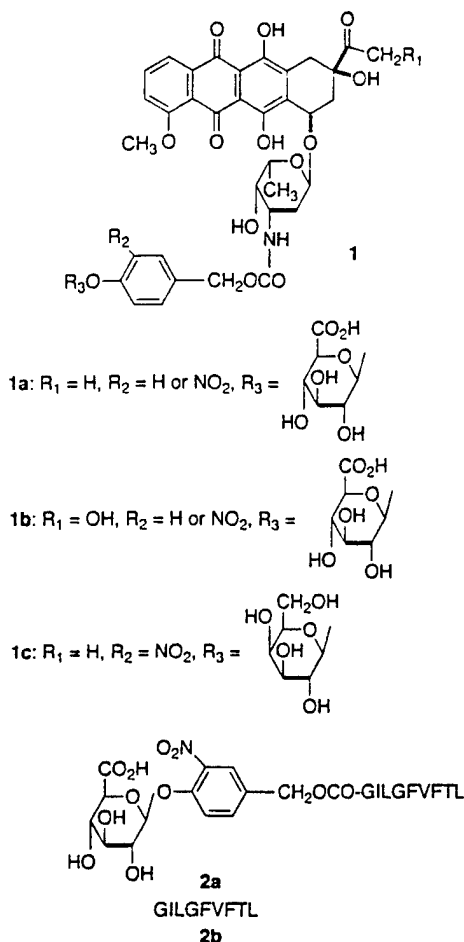
We have chosen a prodrug of nonapeptide GILGFVFTL (2a) as a target of this study. Parent nona-

\* Send correspondence to this author at the Barbara Ann Karmanos Cancer Institute, 110 E. Warren Ave., Detroit, MI 48201-1379. Telephone: (313) 833-0715 x2452. Fax: (313) 832-7294. E-mail: zemlicka@kci.wayne.edu.

<sup>†</sup> Wayne State University School of Medicine.

<sup>‡</sup> Wayne State University.

Chart 1

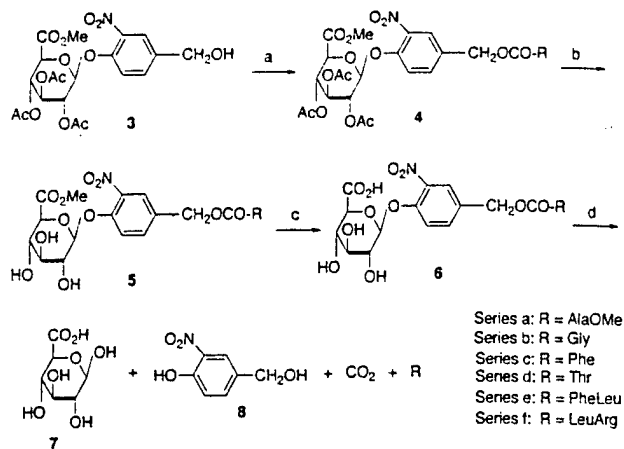


peptide **2b** is an influenza matrix peptide 58–66 which was shown to be optimal for binding to HLA-2 and presentation to cytotoxic T lymphocytes.<sup>17</sup> This article deals with the synthesis and biological investigation of peptide prodrug **2a**.

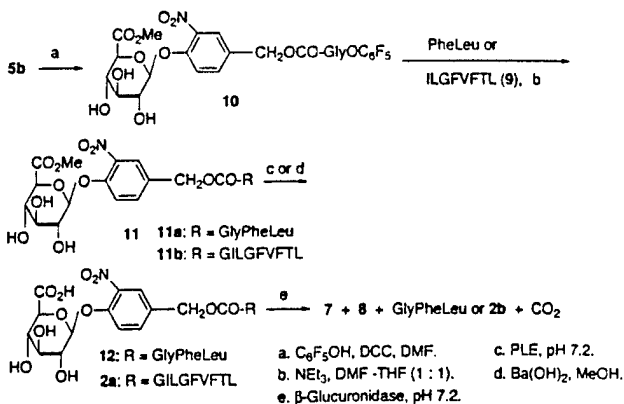
## Synthesis

Although methods for *N*-glycopeptide assembly based on solution- or solid-phase peptide synthesis are available,<sup>18</sup> examples of carbohydrates linked to an *N*-terminus of a peptide through a spacer are rare. In one instance, a  $\beta$ -D-galactopyranosyl-(1 $\rightarrow$ 4)- $\beta$ -D-glucopyranoside moiety was attached through a butyryl spacer to a neutral tricosapeptide in the last step of solid-phase synthesis.<sup>19</sup> Because nonapeptide **2b** is commercially available, a possibility of direct introduction of  $\beta$ -D-glucuronyl-3-nitrobenzyl spacer to the *N*-terminus of **2b** was considered first.

Several model experiments were conducted to determine feasibility of such an approach. Reaction of methyl 2,3,4-tri-*O*-acetyl- $\beta$ -D-glucuronyl-3-nitrobenzyl alcohol (**3**), *N,N'*-disuccinimidyl carbonate (DSC),<sup>8</sup> and AlaOMe in the presence of triethylamine gave smoothly compound **4a** in 62% yield (Scheme 1). In a similar vein, free amino acids Gly, Phe, and Thr were converted to conjugates **4b–4d** in 66–83% yield. Transformation of dipeptides Phe-Leu and Leu-Arg to compounds **4e** and **4f** in 56 and 41% yield, respectively, was also uneventful. Interestingly, in case of Leu-Arg, attachment of the prodrug moiety occurred at the *N*-terminus as evidenced

Scheme 1<sup>a</sup>

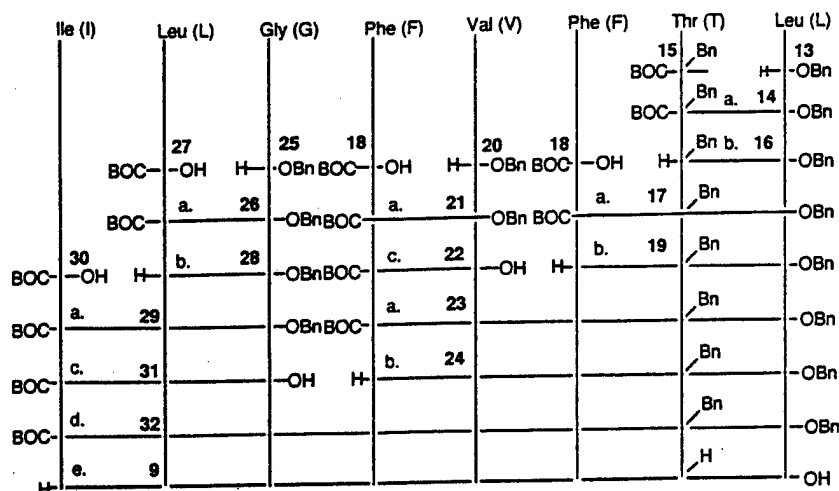
<sup>a</sup> Reagents and conditions: (a) (1) *N,N'*-disuccinyliminocarbonate (DSC), MeCN,  $NEt_3$ , (2)  $R$ ,  $NEt_3$ , DMF; (b) MeONa, MeOH; (c) PLE, pH 7.2; (d)  $\beta$ -glucuronidase, pH 7.2.

Scheme 2<sup>a</sup>

<sup>a</sup> Reagents and conditions: (a)  $C_6F_5OH$ , DCC, DMF; (b)  $NEt_3$ , DMF-THF (1:1); (c) PLE, pH 7.2; (d)  $Ba(OH)_2$ , MeOH; (e)  $\beta$ -glucuronidase, pH 7.2.

by  $^1H$  NMR spectrum and negative reaction with ninhydrin. This can be explained by a protection of arginine moiety (stronger base than triethylamine) by protonation.<sup>20</sup> Deacetylation<sup>8</sup> of conjugates **4b** and **4e** with MeONa in MeOH was also problem-free. Compounds **5b** and **5e** were obtained in 79 and 76% yield, respectively. Methyl glucuronate **5e** was hydrolyzed by pig liver esterase (PLE) to give 91% of glucuronic acid derivative **6e**. The latter compound was quantitatively digested by  $\beta$ -glucuronidase from *Escherichia coli* to give  $\beta$ -glucuronic acid (**7**), alcohol **8**, and Phe-Leu.

Although these experiments have indicated that a direct introduction of a glucuronyl prodrug residue into *N*-termini of amino acids or dipeptides is possible, application to nonapeptide **2b** was beset with problems from the very beginning. Reaction of **2b** with DSC and glucuronyl linker **3** led to formation of a complex mixture of products as shown by HPLC. Although mass spectrum showed that the desired prodrug **2a** was also present, preparative separation would be extremely difficult. Therefore, an alternate synthetic approach was devised. Because unprotected carbohydrate moieties interfere neither with the active ester formation nor peptide bond synthesis,<sup>21,22</sup> it was anticipated that glycine conjugate **5b** can be used for introducing the prodrug function into octapeptide ILGFVFTL (**9**). As an

Scheme 3<sup>a</sup>

<sup>a</sup> Reagents and conditions: (a) DCC, HOBT, THF-DMF, 0 °C; (b) HCO<sub>2</sub>H; (c) H<sub>2</sub>, Pd/C, EtOH; (d) EDCl, HOBT, THF-DMF, 0 °C; (e) (1) H<sub>2</sub>, Pd/C, AcOH, (2) TFA. [EDCl, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride.]

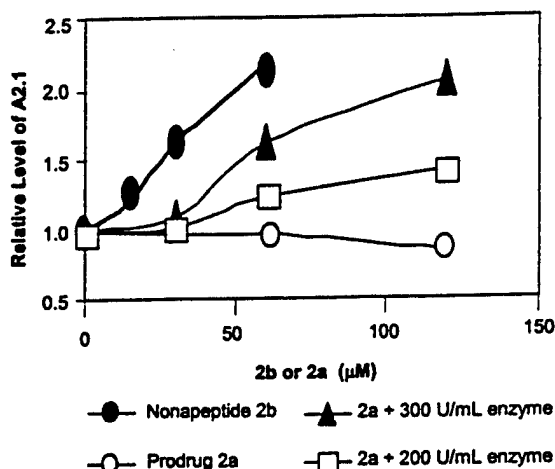
additional advantage, no deprotection of the glucuronate moiety in the latter stages of prodrug synthesis would be necessary. In a model experiment, conjugate 5b was converted to pentafluorophenyl ester 10 (91%, Scheme 2) which was then reacted with PheLeu to give intermediate 11a in 63% yield. Digestion with PLE gave glucuronic acid conjugate 12 (91%). A similar cleavage of glucuronate prodrugs was described.<sup>23,24</sup> Compound 12 was readily degraded by  $\beta$ -glucuronidase from bovine liver to give alcohol 8 and Gly-Phe-Leu.

Next, octapeptide 9 was prepared by a racemization-free fragment coupling strategy<sup>25,26</sup> (Scheme 3). Joining of protected amino acid derivatives 13 and 15 gave dipeptide 14 (84%) which was deprotected to give 16 (87%). Coupling with Boc-Phe-OH (18) gave tripeptide 17 (77%). Deprotection afforded tripeptide fragment 19 (82%). In a similar fashion, Boc-Phe-OH (18) was coupled with Val-OBn (20) to furnish dipeptide 21 (81%). Debenzylation led to 22 (90%) which was converted to pentapeptide 23 by condensation with tripeptide 19 (70%). Deprotection then afforded pentapeptide 24 (90%), a key fragment for synthesis of octapeptide 9. Dipeptide 26 was prepared from Gly-OBn (25) and Boc-Leu-OH (27) in 87% yield. Deprotection of 26 gave dipeptide 28 (85%) which was extended to tripeptide 29 (77%) with Boc-Ile-OH (30). Debenzylation afforded a key tripeptide fragment 31 (85%). Coupling of both fragments 24 and 31 gave octapeptide 32 (66%) which was totally deprotected by hydrogenolysis in AcOH followed by treatment with TFA to give ILGFVFTL (9) in 62% yield.

Condensation of 10 with octapeptide 9 gave the methyl ester of the prodrug 11b (36%). In contrast to dipeptide 5e and tripeptide 11a, the nonapeptide ester prodrug 11b was a poor substrate for PLE. Therefore, ester 11b was hydrolyzed with Ba(OH)<sub>2</sub> in MeOH<sup>24</sup> to give target prodrug 2a (37%). Digestion of 2a with  $\beta$ -glucuronidase from bovine liver gave benzyl alcohol 8 and nonapeptide 2b which were identical with authentic samples.

## Biological Studies

Binding of nonapeptide 2b and prodrug 2a to HLA-A2.1 was investigated with T2 human cells defective in



**Figure 1.** A total of  $3 \times 10^5$  T2 cells were incubated with nonapeptide 2b (solid diamonds) or prodrug 2a (open circles) at concentrations of 15 to 120  $\mu$ M as indicated. *E. coli*  $\beta$ -glucuronidase at 200 (open squares) or 300  $\mu$ M (solid triangles) was added to mixtures containing cells and prodrug 2a. For other details, see Experimental Section.

MHC I-associated peptide processing.<sup>27</sup> Because of the defect, HLA-A2.1 on these cells do not contain endogenous peptides, are unstable, and promptly degraded, resulting in a low level of HLA-A2.1 on the surface. Binding of exogenous peptides to A2.1 gives stable MHC/peptide complexes and increased level of surface HLA-A2.1 which can be detected by flow cytometry.

Nonapeptide 2b binds to T2 cells in a dose-dependent manner in the range of 15 to 60  $\mu$ M. At 60  $\mu$ M of 2b, the HLA-A2.1 level increased to 230% of control cells which were incubated without peptide or  $\beta$ -glucuronidase at pH 6.5–7.0 to approach the physiological conditions. The enzyme alone did not affect the level of HLA-A2.1 (data not shown). Incubation of nonapeptide prodrug 2a up to the 120  $\mu$ M level without  $\beta$ -glucuronidase had no effect on HLA-A2.1, indicating a complete absence of binding (Figure 1). In the presence of 200 and 300 units/mL of  $\beta$ -glucuronidase, HLA-A2.1 level increased with increasing concentration of the enzyme. At 300 units/mL of the enzyme and 120  $\mu$ M of prodrug 2a, the HLA-A2.1 level increased to 210% of the control value. Therefore,  $\beta$ -glucuronidase liberated active

nonapeptide **2b** from prodrug **2b**. Although this pH may not be optimal<sup>28</sup> for the activity of  $\beta$ -glucuronidase in a tumor environment, there is a significant release of active nonapeptide **2b** to mark the cells. Further biological investigations are in progress.

## Conclusion

We have elaborated two basic strategies for introduction of prodrug grouping, which can be activated by  $\beta$ -glucuronidase, into amino acids and peptides. This effort culminated in the synthesis of the first prodrug of antigenic nonapeptide GILGFVFTL (compound **2a**). Nonapeptide **2b** released from **2a** by the action of  $\beta$ -glucuronidase binds to T2 cells in a dose-dependent manner but it is inactive in the absence of the enzyme. This has demonstrated that a prodrug therapy concept previously employed in the context of antitumor anthracycline antibiotics is applicable to exogenous antigenic peptides to mark tumor cells for immune destruction.

## Experimental Section

**General Methods.** NMR spectra were determined at 400 MHz in DMSO-*d*<sub>6</sub>. Optical rotations were determined with a JASCO DIP-370 digital polarimeter. Mass spectrometry was performed in electron-impact (EI) mode on KRATOS MS80, fast-atom bombardment (FAB) mode on KRATOS MS50, or electrospray ionization (ESI) mode on MICROMASS QUATTRO LC-MS. The TLC was run on 6 × 2 cm precoated aluminum sheets of silica gel 60 F<sub>254</sub>, layer thickness 0.2 mm (E. Merck, Darmstadt, Germany), in the following solvents: S<sub>1</sub>, CH<sub>2</sub>Cl<sub>2</sub>-MeOH (9:1); S<sub>2</sub>, CH<sub>2</sub>Cl<sub>2</sub>-MeOH (4:1); S<sub>3</sub>, CH<sub>2</sub>Cl<sub>2</sub>-MeOH-NH<sub>4</sub>OH (6:4:0.1); S<sub>4</sub>, CH<sub>2</sub>Cl<sub>2</sub>-MeOH (3:2); S<sub>5</sub>, CH<sub>2</sub>Cl<sub>2</sub>-THF (4:1); S<sub>6</sub>, CH<sub>2</sub>Cl<sub>2</sub>-MeOH (7:3); S<sub>7</sub>, BuOH-AcOH-H<sub>2</sub>O (8:1:1); and S<sub>8</sub>, CH<sub>2</sub>Cl<sub>2</sub>-MeOH (20:1). HPLC was performed with Waters C-18 column, 3.9 × 300 mm, or Phenomenex Aqua C-18 column, 4.6 × 250 mm, detection at 220 nm, flow rate 0.5 mL/min unless indicated otherwise. With the Waters column the following solvents were used: S<sub>9</sub>, MeCN-H<sub>2</sub>O (3:2, 0.1% TFA); S<sub>10</sub>, MeCN-0.02 M NaH<sub>2</sub>PO<sub>4</sub> (pH 4.0, 2:3); S<sub>11</sub>, MeOH-H<sub>2</sub>O (4:1); S<sub>12</sub>, MeOH-H<sub>2</sub>O (9:1); S<sub>13</sub>, MeOH-H<sub>2</sub>O (65:35); S<sub>14</sub>, MeCN-H<sub>2</sub>O (4:1, 0.1% TFA). Phenomenex column was employed with solvent S<sub>15</sub>, MeCN-H<sub>2</sub>O (1:1, 0.1% TFA), and S<sub>16</sub>, MeCN-H<sub>2</sub>O (45:55, 0.1% TFA). Nonapeptide GILGFVFTL was purchased from Genemed, Inc., San Francisco, CA. Glucuronide **3** was prepared as described.<sup>8</sup> Pig liver esterase (PLE, EC 3.1.1.1),  $\beta$ -glucuronidase (EC 3.2.1.31) from *E. coli* (type X-A), and bovine liver (type B-1) were products of Sigma, St. Louis, MO.

**Methyl N-[(2,3,4-Tri-O-acetyl)- $\beta$ -D-glucopyranuronate-3-nitrobenzyloxycarbonyl]-L-alanine Methyl Ester (4a).** Triethylamine (0.45 mL, 3.29 mmol) was added to a stirred solution of glucuronide **3** (0.5 g, 1.02 mmol) and *N,N'*-disuccinimidyl carbonate (DSC, 0.42 g, 1.64 mmol) in MeCN (5 mL) at room temperature. The progress of reaction was monitored on TLC (CH<sub>2</sub>Cl<sub>2</sub>-THF, 9:1). The starting material **3** was consumed after 2 h. A solution of Al<sub>2</sub>O<sub>3</sub>-HCl (0.15 g, 1.02 mmol) and NEt<sub>3</sub> (0.143 mL, 1.02 mmol) in THF (5 mL) was then added. The mixture was stirred at room temperature for 16 h, solvent was evaporated, and residue was dissolved in CH<sub>2</sub>Cl<sub>2</sub> (150 mL). The organic layer was washed with brine and water (50 mL each), dried (Na<sub>2</sub>SO<sub>4</sub>), and evaporated. Column chromatography on silica gel using solvent S<sub>1</sub> gave product **4a** (0.385 g, 62%): mp 155–156 °C;  $[\alpha]^{24}_D$  36° (c 1, CHCl<sub>3</sub>); TLC, *R*<sub>f</sub> 0.82 (S<sub>1</sub>); HPLC (S<sub>9</sub>, flow rate 1.0 mL/min, *t*<sub>R</sub> 7.98 min, purity 95%); <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$  7.81 (s, 1H), 7.52 (d, 1H) and 7.35 (d, 1H), 7.25 (s, 1H), 5.33 (m, 3H), 5.19 (d, 1H) and 4.19 (d, 1H), 5.09 (s, 2H), 4.36 (m, 1H), 3.74 and 3.75 (2s, 6H), 2.12, 2.06 and 2.05 (3s, 9H), 1.42 (d, 3H); FAB-MS (NaCl) 637 (M + Na). Anal. C<sub>25</sub>H<sub>30</sub>N<sub>2</sub>O<sub>16</sub> (C, H, N).

**Methyl N-[(2,3,4-Tri-O-acetyl)- $\beta$ -D-glucopyranuronate-3-nitrobenzyloxycarbonyl]glycine (4b).** The reaction of glucuronide **3** (0.25 g, 0.51 mmol) with DSC was performed as described for alaninate **4a**. A solution of glycine (38 mg, 0.51 mmol) and NEt<sub>3</sub> (71  $\mu$ L, 0.51 mmol) in DMF (5 mL) was added to the mixture which was stirred at room temperature for 16 h. After evaporation, ethyl acetate (100 mL) was added, and the workup followed the procedure for **4a**. Chromatography using solvent S<sub>2</sub> afforded compound **4b** (0.25 g, 83%): mp 94–96 °C,  $[\alpha]^{25}_D$  15.0° (c 0.1, MeOH); TLC, *R*<sub>f</sub> 0.56 (S<sub>2</sub>); HPLC (S<sub>10</sub>, *t*<sub>R</sub> 10.3 min, purity 90%); <sup>1</sup>H NMR  $\delta$  12.59 (bs, 1H), 7.87 (s, 1H), 7.67–7.57 (m, 2H), 7.40 (d, 1H), 5.72 (d, 1H), 5.43 (t, 1H), 5.11–5.06 (m, 2H), 4.72 (d, 1H), 5.03 (s, 2H), 3.63 (m, 5H), 1.98 (bs, 9H); FAB-MS (KCl) 625 (M + K). Anal. Calcd for C<sub>23</sub>H<sub>26</sub>N<sub>2</sub>O<sub>16</sub>: C, 47.09; H, 4.47; N, 4.78. Found C, 47.15; H, 4.61; N, 4.75.

**Methyl N-[(2,3,4-Tri-O-acetyl)- $\beta$ -D-glucopyranuronate-3-nitrobenzyloxycarbonyl]-L-phenylalanine (4c).** The procedure described for compound **4a** was performed on a 0.41 mmol scale of L-phenylalanine. Chromatography of the crude product using solvent S<sub>2</sub> provided product **4c** (0.175 g, 66%): mp 126–127 °C;  $[\alpha]^{25}_D$  18° (c 0.5, CHCl<sub>3</sub>); TLC, *R*<sub>f</sub> 0.65 (S<sub>2</sub>); HPLC (S<sub>10</sub>, *t*<sub>R</sub> 3.9 min, purity 94%); <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$  7.74 (s, 1H), 7.45 (d, 1H) and 7.32 (d, 1H), 7.26 (bs, 5H), 7.15 (d, 1H), 5.35–5.18 (m, 4H), 4.24 (d, 1H), 5.04 (s, 2H), 4.64 (m, 1H), 3.72 (s, 3H), 3.12 (m, 2), 2.17, 2.11 and 2.05 (3s, 9H); FAB-MS (KCl) 715 (M + K). Anal. C<sub>30</sub>H<sub>32</sub>N<sub>2</sub>O<sub>16</sub> (C, H, N).

**Methyl N-[(2,3,4-Tri-O-acetyl)- $\beta$ -D-glucopyranuronate-3-nitrobenzyloxycarbonyl]-L-threonine (4d).** The reaction was performed on 0.82 mmol scale with L-threonine as described above for compound **4a**. Chromatography using solvent S<sub>6</sub> as eluent gave product **4d** (0.38 g, 73%): mp 90–92 °C;  $[\alpha]^{24}_D$  8.2° (c 0.5, CHCl<sub>3</sub>); TLC, *R*<sub>f</sub> = 0.50 (S<sub>3</sub>); HPLC (S<sub>10</sub>, *t*<sub>R</sub> 10.0 min, purity 82%); <sup>1</sup>H NMR (300 MHz)  $\delta$  7.90 (bd, 1H), 7.68 (d, 1H) and 7.41 (d, 1H), 7.08 (d, 1H), 5.73 (d, 1H), 5.43 (t, 1H) and 5.12–5.02 (m, 4H) and 4.72 (d, 1H), 4.05 (m, 1H), 3.93 (m, 1H), 3.62 (s, 3H), 2.02, 2.01 and 1.98 (3s, 9H), 1.07 (d, 3H); FAB-MS 631 (M + H). Anal. C<sub>25</sub>H<sub>30</sub>N<sub>2</sub>O<sub>17</sub> (C, H, N).

**Methyl N-[(2,3,4-Tri-O-acetyl)- $\beta$ -D-glucopyranuronate-3-nitrobenzyloxycarbonyl]-L-phenylalanyl-L-leucine (4e).** The procedure described above for alaninate **4a** was followed on 2.06 mmol scale of Phe-Leu. Column chromatography on silica gel using solvent S<sub>2</sub> afforded product **4e** (0.9 g, 56%): mp 110–111 °C;  $[\alpha]^{25}_D$  42° (c 1, EtOH); TLC, *R*<sub>f</sub> 0.79 (S<sub>2</sub>); HPLC (S<sub>9</sub>, *t*<sub>R</sub> 13.3, flow rate 1.0 mL/min, purity 90%); <sup>1</sup>H NMR  $\delta$  8.28 (d, 1H), 7.76 (s, 1H) and 7.56 (d, 1H), 7.45 (d, 1H), 7.15 (d, 1H), 7.34–7.18 (m, 5H), 5.72 (d, 1H), 5.44 (t, 1H), 5.10 (m, 2H) and 4.74 (d, 1H), 4.91 (s, 2H), 4.29–4.19 (m, 2H), 3.62 (s, 3H), 3.00–2.64 (m, 2H), 2.06, 1.99 and 1.97 (3s, 9H), 1.62–1.46 (m, 3H), 0.88–0.81 (dd, 6H); FAB-MS 790 (M + H). Anal. C<sub>36</sub>H<sub>43</sub>N<sub>3</sub>O<sub>17</sub> (C, H, N).

**Methyl N-[(2,3,4-Tri-O-acetyl)- $\beta$ -D-glucopyranuronate-3-nitrobenzyloxycarbonyl]-L-leucyl-L-arginine (4f).** The reaction was as described for alaninate **4a** on 0.2 mmol scale of Leu-Arg acetate. Chromatography using solvent S<sub>2</sub> gave product **4f** (67 mg, 40.9%): mp 110°;  $[\alpha]^{24}_D$  8° (c 0.4, CHCl<sub>3</sub>); *R*<sub>f</sub> 0.79 (S<sub>2</sub>); HPLC (S<sub>10</sub>, *t*<sub>R</sub> 7.23 min, purity 84%); <sup>1</sup>H NMR  $\delta$  9.28 (bs, 1H), 7.88 (s, 1H), 7.66 (2d, 3H), 7.44–7.34 (d, 4H), 5.71 (d, 1H), 5.41 (t, 1H), 5.12–4.91 (m, 4H), 4.71 (d, 1H), 3.98 (d, 1H), 3.82 (d, 1H), 3.61 (s, 3H), 2.98 (bs, 2), 2.00, 1.98 and 1.97 (3s, 9H), 1.62–1.38 (m, 7H), 0.79 (dd, 6H); FAB-MS 799 (M + H). Anal. C<sub>33</sub>H<sub>46</sub>N<sub>6</sub>O<sub>17</sub>·2H<sub>2</sub>O (C, H, N, -0.44).

**Methyl N- $\beta$ -D-Glucopyranuronate-3-nitrobenzyloxycarbonyl]glycine (5b).** Freshly prepared MeONa (0.79 mmol) in MeOH (2 mL) was added with stirring to a solution of compound **4b** (0.5 g, 0.63 mmol) in MeOH (20 mL) at 0 °C. The progress of deacetylation was monitored by TLC (S<sub>4</sub>) and HPLC (S<sub>10</sub>, flow rate 0.8 mL/min). The starting material was consumed in 30 min. Dowex 50 (H<sup>+</sup>) form, 0.25 g) was added, the mixture was filtered, and solvent was evaporated. Chromatography of the residue using solvent S<sub>4</sub> afforded product **5b** (0.320 g, 79%): mp 88–89 °C;  $[\alpha]^{25}_D$  -25° (c 0.1, MeOH); TLC, *R*<sub>f</sub> 0.50 (S<sub>3</sub>); HPLC (S<sub>15</sub>, *t*<sub>R</sub> 5.3 min, purity 99%); <sup>1</sup>H NMR

$\delta$  7.88 (s, 1H), 7.66 (d, 1H) and 7.52 (d, 1H), 7.15 (bs, 1H), 6.05 (d, 1H), 5.86 (d, 1H), 4.03 (t, 1H), 3.78 (t, 1H), 3.48 (d, 1H), 5.02 (s, 2H), 3.68 (s, 3H), 3.42–3.35 (m, 6H); ESI-MS (NaCl) 483 (M + Na). Anal.  $C_{17}H_{20}N_2O_{13} \cdot H_2O$  (C, H, N).

**Methyl *N*-[ $\beta$ -D-Glucopyranuronate-3-nitrobenzyloxy-carbonyl]-L-phenylalanyl-L-leucine (5e).** The reaction with triacetate 4e was performed as described for compound 5b to give product 5e (0.32 g, 76%); mp 142–143 °C;  $[\alpha]^{25}_D$  –38.8° (c 0.5, EtOH); TLC,  $R_f$  0.51 ( $S_3$ ); HPLC ( $S_{10}$ , flow rate 0.8 mL/min,  $t_R$  10.1 min, purity 85%);  $^1H$  NMR  $\delta$  8.26 (d, 1H), 7.74 (s, 1H) and 7.53 (d, 1H), 7.44 and 7.37 (2d, 2H), 7.29–7.12 (m, 5H), 5.53–5.49 (3 overlapped s, 3H), 4.90 (s, 2H), 5.29 (apparent d, 2H), 4.12 (d, 1H), 4.29–4.19 (m, 2H), 3.63 (s, 3H), 3.00–2.57 (m, 2H), 1.6–1.46 (m, 3H), 0.89–0.81 (dd, 6H); FAB-MS (KCl) 702 (M + K). Anal.  $C_{30}H_{37}N_3O_{14}$  (C, H, N).

***N*-[ $\beta$ -D-(Glucopyranuronic acid)-3-nitrobenzyloxy-carbonyl]-L-phenylalanyl-L-leucine (6e).** Compound 5e (14.5 mg, 21  $\mu$ mol) in 0.02 M  $Na_2HPO_4$  (pH 7.2, 10 mL) was incubated with PLE (1,200 units) at 37 °C. Progress of the reaction was monitored by HPLC (MeCN– $NaH_2PO_4$ , pH 4.0, 3:7, flow rate 0.6 mL/min). The starting material was consumed in 1 h with the formation of a new peak ( $t_R$  5.6 min). The pH was adjusted to 4.0 with HCl, and the mixture was extracted with ethyl acetate. The organic phase was washed with water, it was dried ( $Na_2SO_4$ ), and the solvent was evaporated to give compound 6e (13 mg, 91%); mp 122–124 °C;  $[\alpha]^{25}_D$  –14° (c 0.25, EtOH); TLC,  $R_f$  0.42 ( $S_3$ ); HPLC ( $S_{10}$ , flow rate 0.8 mL/min,  $t_R$  5.6 min, purity 95%);  $^1H$  NMR  $\delta$  8.25 (d, 1H), 7.74 (s, 1H) and 7.53 (d, 1H), 7.43 (d, 1H) and 7.34 (d, 1H), 7.27–7.15 (m, 5H), 5.48 (d, 1H), 4.42 (d, 1H), 3.94 (m, 2H) and 3.25 (d, 1H), 5.24 (3 overlapped s, 3H), 4.90 (s, 2H), 4.24 (m, 2), 3.00–2.96 (m, 2H), 1.65–1.49 (m, 3H), 0.84 (dd, 6H); FAB-MS (KCl) 688 (M + K). Anal.  $C_{29}H_{35}N_3O_{14}$  (C, H, N).

**Digestion of Compound 6e with  $\beta$ -Glucuronidase.** Compound 6e (1 mg, 1.5  $\mu$ mol) and *E. coli*  $\beta$ -glucuronidase (800 units) were incubated in 0.02 M  $Na_2HPO_4$  (pH 7.2, 1 mL) at 37 °C. Progress of reaction was monitored by HPLC/MeCN–0.02 M  $NaH_2PO_4$  (pH 4.0) 3:7, flow rate 0.6 mL/min). The starting material was consumed within 1 h with the formation of Phe-Leu and 4-hydroxy-3-nitrobenzyl alcohol (8) whose  $t_R$ 's (4.8 and 9.7 min, respectively) were identical with those of the corresponding authentic samples.

**Methyl *N*-[ $\beta$ -D-Glucopyranuronate-3-nitrobenzyloxy-carbonyl]glycine Pentafluorophenyl Ester (10).** DCC (0.138 g, 0.67 mmol) and pentafluorophenol (0.124 g, 0.67 mmol) were added to a solution of compound 5b (0.31 g, 0.67 mmol) in DMF–THF (1:5, 30 mL) at 0 °C. The mixture was stirred for 2 h at 0 °C and then at room temperature for 16 h. The solvents were evaporated, the residue was dissolved in EtOAc (50 mL), the solution was filtered, and the filtrate was evaporated. Chromatography on a silica gel column using hexane:EtOAc (1:4) as eluent gave 0.385 g (91%) of product 10, which was used for the next coupling reaction: mp 145–147 °C;  $[\alpha]^{25}_D$  –31° (c 0.1, MeOH); TLC,  $R_f$  0.8 ( $S_5$ ); HPLC ( $S_{11}$ , flow rate 0.8 mL/min,  $t_R$  4.8 min, purity 84%);  $^1H$  NMR  $\delta$  7.80 (s, 1H), 7.59 (d, 1H) and 7.38 (d, 1H), 5.26 (d, 1H), 4.24 (bs, 2H), 4.07 (d, 1H), 3.04 (m, 3H), 5.02 (s, 2H), 3.61 (s, 3H), 3.36–3.24 (m, 3H); FAB-MS (NaCl) 649 (M + Na).

**Methyl *N*-[ $\beta$ -D-Glucopyranuronon-3-nitrobenzyloxy-carbonyl]glycyl-L-phenylalanyl-L-leucine (11a).** A solution of pentafluorophenyl ester 10 (0.1 g, 0.159 mmol) in THF (5 mL) was added with stirring to Phe-Leu (45 mg, 0.159 mmol) and  $NEt_3$  (25  $\mu$ L, 0.159 mmol) in DMF (5 mL) at room temperature. The stirring was continued for 16 h, the solvents were evaporated, and residue was chromatographed on a silica gel column using solvent  $S_8$  to give compound 11a (72 mg, 63%); mp 160–161 °C,  $[\alpha]^{25}_D$  –27° (c 0.1, MeOH); TLC,  $R_f$  0.50 ( $S_4$ ); HPLC ( $S_{10}$ ,  $t_R$  11.0 min, purity 93%);  $^1H$  NMR  $\delta$  8.24 (d, 1H), 8.01 (d, 1H), 7.82 (s, 1H), 7.59 (d, 1H), 7.43 (m, 2H), 7.19 (m, 5H), 5.50 (bs, 2H), 5.30 (d, 2H), 4.10 (d, 1H), 3.57 (d, 1H), 3.52 (bs, 1H), 4.98 (s, 2H), 4.53 (q, 1H), 4.35 (bs, 1H), 4.16 (q, 1H), 3.62 (s, 3H), 3.38 (bs, 2H), 3.00 (dd, 2H), 2.72 (m, 1H),

1.51–1.61 (m, 3H), 0.85 (dd, 6H); ESI-MS (NaCl) 743 (M + Na).

***N*-[ $\beta$ -D-(Glucopyranuronic acid)-3-nitrobenzyloxy-carbonyl]glycyl-L-phenylalanyl-L-leucine (12).** Compound 11a (20 mg, 28  $\mu$ mol) and PLE (1,200 units) were incubated in  $Na_2HPO_4$  (0.02 M, pH 7.2, 10 mL) at 37 °C. Progress of reaction was monitored by HPLC ( $S_{10}$ , flow rate 0.4 mL/min). The starting material was consumed in 1 h with the formation of a new major peak with  $t_R$  7.2. The pH was adjusted to 4.0 with HCl, the mixture was partitioned between ethyl acetate and water, and the organic phase was dried ( $Na_2SO_4$ ) and evaporated: yield 18 mg (91%) of tripeptide derivative 12; mp 171–173 °C;  $[\alpha]^{25}_D$  –14° (c 0.1, MeOH); TLC,  $R_f$  0.49 ( $S_3$ ); HPLC ( $S_{10}$ , flow rate 0.6 mL/min,  $t_R$  7.4 min, purity 95%);  $^1H$  NMR  $\delta$  12.62 (bs, 1H), 8.28 (d, 1H), 8.00 (d, 1H), 7.83 (s, 1H), 7.59 (d, 1H) and 7.43 (m, 2H), 7.18 (m, 5H), 5.49 (bs, 1H), 5.25 (bs, 2H), 4.98 (s, 2H), 4.54 (q, 1H), 4.21 (q, 1H), 4.01–3.92 (m, 2H), 3.62–3.44 (m, 3H), 3.00 (dd, 2H), 2.72 (m, 1H), 1.51–1.61 (m, 3), 0.85 (dd, 6H); ESI-MS (NaCl) 729 (M + Na). Anal.  $C_{31}H_{38}N_4O_{15}$  (C, H, N).

**Digestion of Compound 12 by  $\beta$ -Glucuronidase.** Compound 12 (1 mg, 1.4  $\mu$ mol) was incubated with bovine liver  $\beta$ -glucuronidase (800 units) in phosphate buffer (0.02 M, pH 7.2, 1 mL) at 37 °C. The reaction was followed by HPLC ( $S_{10}$ , flow rate 0.6 mL/min). The starting material was consumed in 4 h with the formation of two major peaks at  $t_R$  6.5 (Gly-Phe-Leu) and 14.0 (compound 8).

**H-Leu-OBn (13).** Free base 13 was released from the commercially available tosylate using  $NaHCO_3$  (pH 8.0) and extracted with ethyl acetate as a thick oil (5.1 g, 92%);  $R_f$  0.6 ( $S_7$ );  $^1H$  NMR  $\delta$  7.34 (bs, 5H), 5.07 (bs, 2H), 3.30 (bs, 1H), 1.71–1.66 (m, 3H), 1.40–1.29 (m, 2H), 0.82 (m, 6H); EI-MS 221 (M + H).

**Boc-Thr(Bn)-Leu-OBn (14).** HOBT (2.19 g, 16.16 mmol) and DCC (3.32 g, 16.16 mmol) were added with stirring to a solution of Boc-Thr(Bn)-OH (15, 5 g, 16.16 mmol) in THF (50 mL) at 0 °C. The stirring was continued for 30 min. The H-Leu-OBn (13, 4.46 g, 20.20 mmol) in DMF (30 mL) cooled to 0 °C was then added, and the mixture was stirred for 2 h and at room temperature for 16 h. The solvents were evaporated, the residue was dissolved in EtOAc (125 mL), and dicyclohexyl urea (DCU) was filtered off. The filtrate was washed twice with saturated aqueous  $NaHCO_3$ , 10% citric acid, and water (50 mL each); it was dried ( $Na_2SO_4$ ) and evaporated. Chromatography using solvent  $S_8$  as eluent gave dipeptide 14 (7.0 g, 84%); mp 84–85 °C,  $R_f$  0.62 ( $S_8$ );  $^1H$  NMR  $\delta$  8.24 (d, 1H) and 6.59 (d, 1H), 7.33–7.24 (m, 10H), 5.07 (bs, 2H) and 4.35 (m, 2H), 4.49 (m, 1H), 4.05 (m, 1H), 3.79 (t, 1H), 1.58–1.47 (m, 3H), 1.36 (s, 9H), 1.07 (d, 3H), 0.78 (dd, 6H); EI-MS 512 (M).

**H-Thr(Bn)-Leu-OBn (16).** A solution of dipeptide 14 (6.5 g, 12.69 mmol) in  $HCO_2H$  (97%, 50 mL) was stirred at room temperature for 6 h. The solvent was evaporated, residue was dissolved in water (150 mL), and solution was extracted with ether (2  $\times$  50 mL). The pH of the aqueous phase was adjusted to 8.0 with aqueous  $NaHCO_3$  and extracted with EtOAc (2  $\times$  100 mL). The organic portion was washed with water (50 mL), and it was dried ( $Na_2SO_4$ ) and evaporated to give dipeptide 16 as a thick oil (4.6 g, 87%);  $R_f$  0.72 ( $S_2$ );  $^1H$  NMR  $\delta$  8.56 (bs, 1H), 7.30 (2s, 10H), 5.09 (s, 2H), 4.50–4.35 (m, 4H), 3.81 (s, 1H), 1.58 (bs, 3H), 1.15–1.14 (d, 3H), 0.81 (dd, 6H); FAB-MS 413 (M + H).

**Boc-Phe-Thr(Bn)-Leu-OBn (17).** The reaction was performed as described for dipeptide 14 using Boc-Phe-OH (18, 3.66 g, 13.8 mmol), HOBT (1.87 g, 13.8 mmol), and DCC (2.84 g, 13.8 mmol) in THF (50 mL). Dipeptide 16 (3.8 g, 9.21 mmol) was then added in DMF solution (20 mL), and the mixture was stirred for 2 days at room temperature and worked-up as described for dipeptide 14. Chromatography in  $CH_2Cl_2$ :MeOH (10:1) furnished tripeptide 17 (4.7 g, 77%); mp 150–152 °C;  $R_f$  0.64 ( $S_1$ );  $^1H$  NMR  $\delta$  8.24 (d, 1H), 7.82 (d, 1H) and 7.09 (d, 1H), 7.27 (m, 15H), 5.05 (dd, 2H), 4.48–4.35 (m, 4H), 4.23 (m, 1H), 3.90 (t, 1H), 2.95 (m, 1H), 2.71 (m, 1H), 1.54 (m, 3H), 1.25 (s, 9H), 1.08 (d, 3H), 0.82 (dd, 6H); FAB-MS (KCl) 698 (M + K).



**H-Phe-Thr(Bn)-Leu-OBn (19).** It was prepared as described for dipeptide 16 from tripeptide 17 (4 g, 6.06 mmol). The tripeptide 19 (2.8 g, 82%) was obtained as a thick oil: TLC,  $R_f$  0.71 ( $S_2$ ); HPLC ( $S_{12}$ , flow rate 0.6 mL/min,  $t_R$  6.6 min, 96% purity);  $^1H$  NMR  $\delta$  8.32 (d, 1H), 8.27 (d, 1H), 7.31–7.20 (m, 15H), 5.06 (dd, 2H), 4.47–4.36 (m, 5H), 3.90 (t, 1H), 3.69 (bs, 1H), 3.02 (m, 1H), 2.68 (m, 1H), 1.58–1.50 (m, 4H), 1.03 (d, 3H), 0.83 (dd, 6H); ESI-MS 560 (M + H).

**H-Val-OBn (20).** Free base was released from the corresponding hydrochloride as described for H-Leu-OBn (13) as a thick oil:  $R_f$  0.7 ( $S_7$ );  $^1H$  NMR  $\delta$  7.33 (m, 5H), 5.12 (dd, 2H), 3.13 (d, 1H), 1.83 (m, 1H), 1.69 (bs, 2H), 0.80 (dd, 6H); EI-MS 207 (M + H).

**Boc-Phe-Val-OBn (21).** The reaction was performed as described for dipeptide 14 with Boc-Phe-OH (18, 5.0 g, 18.84 mmol) and H-Val-OBn (20, 5.49 g, 26.35 mmol) to give dipeptide 21 (7 g, 81%); mp 68–69 °C;  $R_f$  0.48 ( $S_8$ );  $^1H$  NMR  $\delta$  8.17 (d, 1H), 7.38 (m, 10H), 6.95 (d, 1H), 5.11 (dd, 2H), 4.24 (t, 2H), 2.88 (m, 1H), 2.67 (t, 1H), 2.06 (m, 1H), 1.26 (s, 9H), 0.86 (m, 6H); EI-MS 454 (M).

**Boc-Phe-Val-OH (22).** Boc-Phe-Val-OBn (21, 4.0 g, 8.81 mmol) was hydrogenated in a Parr apparatus at 40 psi for 2 h in ethanol (50 mL) over Pd-C (10%, 0.42 g). The catalyst was filtered off, and the solvent was evaporated. The residue was partitioned between aqueous  $NaHCO_3$  (100 mL) and ether (2  $\times$  50 mL). The mixture (pH 3) was extracted with ethyl acetate (2  $\times$  100 mL), and the organic phase was washed with water, dried ( $Na_2SO_4$ ), and evaporated to give the dipeptide 22 (2.9 g, 90%); mp 77–78 °C;  $R_f$  0.58 ( $S_7$ ); HPLC ( $S_{12}$ , flow rate 0.6 mL/min,  $t_R$  5.6 min, purity 97%);  $^1H$  NMR  $\delta$  7.88 (d, 1H), 7.23 (m, 5H), 6.96 (d, 1H), 4.23–4.14 (m, 2H), 2.93 (m, 1H), 2.71 (q, 1H), 2.05 (m, 1H), 1.26 (s, 9H), 0.87 (m, 6H); FAB-MS (KCl) 403 (M + K).

**Boc-Phe-Val-Phe-Thr(Bn)-Leu-OBn (23).** The reaction of dipeptide 22 (2.5 g, 7.01 mmol) with tripeptide 19 (2.8 g, 5.00 mmol) was performed as described for dipeptide 14. Chromatography in solvent ( $S_8$ ) furnished pentapeptide 23 (4.4 g, 70%); mp 80–81 °C;  $R_f$  0.66 ( $S_2$ ), HPLC ( $S_{14}$ , flow rate 0.6 mL/min,  $t_R$  11.4 min, purity 90%);  $^1H$  NMR  $\delta$  8.19 (d, 1H), 8.12 (d, 1H), 8.02 (d, 1H) and 7.61 (d, 1H), 7.32–7.10 (m, 20H), 7.00 (d, 1H), 5.00 (dd, 2H), 4.70 (m, 1H), 4.47–4.36 (m, 6H), 4.21–4.11 (m, 2H), 3.89 (t, 1H), 3.00 (m, 1H), 2.87–2.63 (m, 2H), 1.90 (m, 1H), 1.53 (m, 2H), 1.25 (s, 9H), 1.05 (d, 3H), 0.82 (dd, 6H), 0.73 (m, 6H); ESI-MS (NaCl) 928 (M + Na).

**H-Phe-Val-Phe-Thr(Bn)-Leu-OBn (24).** Deprotection of pentapeptide 23 (0.5 g, 0.55 mmol) was performed as described for dipeptide 14 to afford free pentapeptide 24 (0.4 g, 90%); mp 147–148 °C;  $R_f$  0.55 ( $S_2$ ); HPLC ( $S_{14}$ , flow rate 0.6 mL/min,  $t_R$  6.3 min, purity 96%);  $^1H$  NMR  $\delta$  8.25 (d, 1H), 8.14 (d, 1H), 8.02 (d, 1H) and 7.89 (d, 1H), 7.30–7.17 (m, 20H), 5.05 (dd, 2H), 4.68 (m, 1H), 4.47–4.36 (m, 6H), 4.16 (m, 2H), 3.90 (t, 1H), 2.95 (m, 1H), 2.75 (m, 2H), 1.89 (m, 1H), 1.52 (m, 4H), 1.05 (d, 3H), 0.82 (dd, 6H), 0.75–0.63 (dd, 6H); ESI-MS 806 (M + H).

**H-Gly-OBn (25).** Free base was released from the corresponding hydrochloride as described in the workup procedure for dipeptide 16 as a thick oil:  $R_f$  0.50 ( $S_1$ );  $^1H$  NMR  $\delta$  7.38–7.30 (m, 5H), 5.11 (s, 2H), 3.87 (m, 2H), 1.89 (bs, 2H); ESI-MS 165 (M + H).

**Boc-Leu-Gly-OBn (26).** It was prepared as described for dipeptide 14 from Boc-Leu-OH (27, 5 g, 20.05 mmol) and Gly-OBn (25, 4.13 g, 25.06 mmol). Chromatography afforded dipeptide 26 as a thick oil (7.0 g, 87%);  $R_f$  0.90 ( $S_8$ );  $^1H$  NMR  $\delta$  8.26 (t, 1H), 7.32 (m, 5H), 6.89 (d, 1H), 5.09 (s, 2H), 3.91–3.82 (m, 3H), 1.60 (m, 1H), 1.34 (s, 11H), 0.81 (m, 6H); FAB-MS (KCl) 417 (M + K).

**H-Leu-Gly-OBn (28).** Deprotection of 26 (8 g, 21.16 mmol) was performed as described for dipeptide 14 to give 28 (5.88 g, 85%);  $R_f$  0.51 ( $S_6$ );  $^1H$  NMR  $\delta$  8.60 (bs, 1H), 7.34 (s, 5H), 5.10 (s, 2H), 3.91 (ddd, 2H), 3.40 (t, 1H), 1.69 (bs, 2H), 1.44 (m, 1H), 1.30 (m, 2H), 0.83 (m, 6H); ESI-MS 279 (M + H).

**Boc-Ile-Leu-Gly-OBn (29).** This tripeptide was prepared as described for dipeptide 14 from Boc-Ile-OH (30, 2.50 g, 10.80 mmol) and dipeptide 28 (2.5 g, 8.99 mmol). Chromatography

gave tripeptide 29 (5.31 g, 77%); mp 50–51 °C;  $R_f$  0.62 ( $S_8$ );  $^1H$  NMR  $\delta$  8.40 (bs, 1H), 7.79 (d, 1H), 7.33 (s, 5H), 6.80 (d, 1H), 5.08 (s, 2H), 4.36 (m, 1H), 3.94–3.73 (m, 3H), 1.62 (bs, 2H), 1.34 (s, 9H), 1.04 (m, 2H), 0.83–0.77 (m, 14H); ESI-MS 492 (M + H).

**Boc-Ile-Leu-Gly-OH (31).** Tripeptide 29 (2.0 g, 4.07 mmol) was hydrogenated in ethanol (25 mL) over Pd-C (10%, 0.2 g) as described for dipeptide 22 to give tripeptide 31 (1.4 g, 85%); mp 108–109 °C;  $R_f$  0.69 ( $S_1$ ); HPLC ( $S_{13}$ , flow rate 0.8 mL/min,  $t_R$  7.9, purity 98.5%);  $^1H$  NMR  $\delta$  8.18 (bs, 1H), 7.79 (d, 1H), 6.82 (d, 1H), 4.36 (m, 1H), 3.77–3.62 (m, 3H), 1.63 (bs, 2H), 1.34 (s, 9H), 1.03 (m, 2H), 0.85–0.77 (m, 14H); ESI-MS 402 (M + H).

**Boc-Ile-Leu-Gly-Phe-Val-Phe-Thr(Bn)-Leu-OBn (32).** To a solution of tripeptide 31 (0.460 g, 1.02 mmol) in THF (20 mL) was added HOBT (0.14 g, 1.02 mmol) at 0 °C followed by 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI, 0.196 g, 1.02 mmol). The reaction mixture was stirred for 30 min. A cooled solution of pentapeptide 24 (0.46 g, 0.57 mmol) in DMF (10 mL) was then added. The stirring was continued at 0 °C for 2 h and at room temperature for 3 days. The mixture was worked up as described for the dipeptide 14. Chromatography of the crude product in solvent  $S_8$  provided octapeptide 32 (0.45 g, 66%); mp 177–179 °C;  $R_f$  0.57 ( $S_8$ ); HPLC ( $S_{14}$ , flow rate 0.6 mL/min,  $t_R$  9.20, 91% purity);  $^1H$  NMR  $\delta$  8.11 (t, 2H), 8.03 (d, 1H), 7.98 (t, 1H), 7.92 (bs, 2H), 7.79 (d, 1H), 7.32–7.11 (m, 20H), 6.78 (d, 1H), 5.05 (dd, 2H), 4.70 (m, 1H), 4.55 (m, 1H), 4.47–4.36 (m, 4H), 4.27 (m, 1H), 4.13 (m, 1H), 3.89 (m, 2H), 3.59 (m, 2H), 2.80 (m, 4H), 1.34 (s, 9H), 1.56 (m, 3H), 1.05 (d, 3H), 0.83–0.77 (m, 26H); ESI-MS (NaCl) 1,211 (M + Na).

**H-Ile-Leu-Gly-Phe-Val-Phe-Thr-Leu-OH (9).** Octapeptide 32 (0.2 g, 4.07 mmol) was hydrogenated in AcOH (50 mL) over Pd-C (10%, 0.4 g) for 6 h. The catalyst was filtered off and the solvent evaporated. The residue was dissolved in TFA (10 mL), and the solution was stirred at room temperature for 1 h. The solvent was evaporated, and residue (0.14 g) was purified by HPLC using Phenomenex Aqua semipreparative column (10  $\times$  250 mm,  $S_{16}$ , flow rate 2.4 mL/min,  $t_R$  7.4 min) to give octapeptide 9 (95 mg, 62%); mp 124–126 °C;  $R_f$  0.63 ( $S_7$ ); HPLC ( $S_{15}$ ,  $t_R$  4.2 min, flow rate 0.8 mL/min, purity 92%);  $^1H$  NMR  $\delta$  12.60 (bs, 1H), 8.43 (bs, 1H), 8.29 (d, 1H), 8.20 (bs, 1H), 8.07 (bs, 3H), 7.86 (bs, 1H), 7.20–7.14 (b, 10H), 4.79 (d, 1H), 4.61 (bs, 1H), 4.38 (m, 2H), 4.24–4.16 (m, 3H), 3.90 (bs, 1H), 3.76 (m, 1H), 3.61 (bs, 2H), 2.98 (m, 2H), 2.75 (m, 2H), 1.90 (m, 1H), 1.75 (m, 1H), 1.61–1.43 (m, 6H), 1.01 (bs, 3H), 0.86–0.72 (m, 26H); ESI-MS 909 (M + H).

**Methyl N-[ $\beta$ -D-Glucopyranuronate-3-nitrobenzyloxy-carbonyl]-Gly-Ile-Leu-Gly-Phe-Val-Phe-Thr-Leu-OH (11b).** A solution of pentafluorophenylester 10 (136 mg, 0.22 mmol) in THF (10 mL) was added to a mixture of octapeptide 9 (100 mg, 0.11 mmol) and  $NEt_3$  (15  $\mu$ L, 0.11 mmol) in DMF (10 mL) with stirring at room temperature. The stirring was continued for 40 h, the solvents were evaporated, and residue was chromatographed (three times) using  $CHCl_3$ :MeOH (3:2) to obtain compound 11b (68 mg, 46%) which was further purified by semipreparative HPLC (see octapeptide 9,  $S_{16}$ , flow rate 3.4 mL/min) to give 52 mg (34%) of 11b: HPLC ( $S_{16}$ , flow rate 1.0 mL/min,  $t_R$  13.1, purity 86%); mp 140–142 °C;  $[\alpha]_D^{25}$  11° (c 0.1, MeOH);  $R_f$  0.49 ( $CHCl_3$ :MeOH: $NH_4OH$  (4:6:0.1));  $^1H$  NMR  $\delta$  8.11 (bs, 4H), 8.03 (bs, 1H), 7.98 (d, 1H), 7.93 (d, 1H), 7.88 (d, 1H), 7.83 (bs, 1H), 7.61 (d, 1H), 7.53 (bs, 1H), 7.41 (d, 1H), 7.26–7.11 (m, 10H), 5.54–5.50 (m, 2H), 5.30 (m, 2H), 4.99 (s, 2H), 4.66 and 4.52 (2m, 3H), 4.35 (bs, 1H), 4.25 (bs, 2H), 4.14–4.00 (m, 5H), 3.63 (s, 3H), 3.54 (d, 1H), 3.15 (d, 1H), 3.05 (m, 5H), 2.88–2.60 (m, 7H), 1.86 (m, 1H), 1.63 (m, 1H), 1.54 (m, 1H), 1.39 (m, 2H), 1.03 (m, 3H), 0.98 (d, 3H), 0.86–0.74 (m, 23H); Negative ESI-MS ( $NH_4OH$ ) 1,349 (M – H).

**N-[ $\beta$ -D-(Glucopyranuronic acid)-3-nitrobenzyloxy-carbonyl]-Gly-Ile-Leu-Gly-Phe-Val-Phe-Thr-Leu-OH (2a).** A mixture of compound 11b (20 mg, 0.014 mmol) and  $Ba(OH)_2 \cdot 8H_2O$  (4.6 mg, 0.014 mmol) in MeOH (10 mL) was stirred at room temperature for 3 h. Dowex 50 ( $H^+$ ) form, 25 mg) was then added, and the mixture was filtered. The filtrate was evapo-

rated to give crude prodrug which was purified by semi-preparative HPLC (see octapeptide 9, flow rate 3.4 mL/min) to give 9 mg (47%) of 2a. Analytical HPLC (flow rate 1.0 mL/min,  $t_R$  7.9 min, purity 76%): mp 180–181 °C,  $[\alpha]_D^{25}$  6.0° (c 0.1, MeOH);  $^1H$  NMR (500 MHz)  $\delta$  8.07 (d, 1H), 8.01 (d, 1H), 7.96–7.91 (m, 4H), 7.87 (d, 1H), 7.84 (s, 1H), 7.81 (d, 1H), 7.61 (d, 1H), 7.51 (bs, 1H), 7.42 (d, 1H), 7.25–7.31 (m, 10H), 5.48 (bs, 1H), 5.40 (bs, 1H), 5.26 (d, 2H), 5.00 (s, 2H), 4.78 (bs, 1H), 4.67 and 4.55 (2m, 4H), 4.24–4.12 (m, 6H), 3.96–3.91 (m, 2H), 3.60 (m, 2H), 3.03 (m, 2H), 2.89 (m, 2H), 2.79 (m, 2H), 2.66 (m, 2H), 2.53 (bs, 5H), 1.90 (m, 1H), 1.66 (m, 2H), 1.41 (m, 2H), 1.22 (bs, 3H), 1.03 (d, 3H), 0.88–0.74 (m, 26H); Negative ESI-MS (NH<sub>4</sub>OH) 1335 (M – H).

**Hydrolysis of Peptide Prodrug 2a by  $\beta$ -Glucuronidase.** The hydrolysis of propeptide 2a was performed as described for propeptide 12. It was followed by HPLC ( $S_{18}$ , flow rate 1.0 mL/min). The starting material was consumed in 4 h with the formation of two peaks with  $t_R$  3.6 and 4.5 min, corresponding to the authentic samples of 4-hydroxy-3-nitrobenzyl alcohol (8) and nonapeptide 2b, respectively.

**Binding of Nonapeptide 2b and Prodrug 2a to HLA-A2.1:** T2 cells were maintained in AIM V medium (GIBCO BRL/Life Technologies, Gaithersburg, MD) containing 10% fetal calf serum. A total of  $3 \times 10^5$  T2 cells were incubated in the medium with varying concentrations of 2b or 2a. To activate the prodrug 2a,  $\beta$ -glucuronidase from *E. coli* (200 and 300 units/mL) was added to the cell culture. The pH was maintained at  $6.6 \pm 0.2$  in this medium. The mixtures were incubated at 37 °C for 1 h and then at 28 °C overnight. To measure the level of HLA-A2.1, cells were stained with mouse monoclonal antibody BB7.2 (American Type Culture Collection). Unbound antibody was removed by extensive washing, and bound antibody was visualized by staining with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Jackson Immunological Research Laboratory, West Grove, PA). Isotype matched monoclonal antibody was the negative control. Flow cytometric analysis was performed with a FACS Calibur (Beckton Dickinson, San Jose, CA), and the data are recorded as mean channel fluorescence. The results (average from three separate experiments) are given in Figure 1.

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